IDENTIFICATION AND CHARACTERIZATION OF THE FUNCTIONAL DOMAINS OF DIPTHERIA TOXIN REPRESSOR (DTXR)

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ABSTRACT

Title of Dissertation:

Identification and Characterization of Functional Domains of the Diphtheria Toxin Repressor (DtxR)

Zhaoxi Wang

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Dissertation directed by:

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Corynebacterium diphtheriae is the causative agent of diphtheria. Diphtheria toxin, a potent bacterial exotoxin encoded by the *tox* gene of certain temperate corynebacteriophages, is the primary virulence factor of *C. diphtheriae*. In *C. diphtheriae*, the production of diphtheria toxin and the iron uptake system are negatively controlled by the diphtheria toxin repressor (DtxR), an iron-dependent metalloregulatory protein. The regulatory function of DtxR can be activated by various divalent cations, including Fe^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}.

In this investigation, we focused on understanding the structural basis of metalloregulation of DtxR. First, we identified and characterized 20 distinct mutations

in *dtxR* induced by random mutagenesis with bisulfite treatment, eighteen of which caused single amino acid substitutions in DtxR and two of which were chain-terminating mutations. Six of the amino acid replacements were clustered between residues 39 and 52 in a predicted helix-turn-helix motif that exhibits homology with several other repressors and is identified as the putative DNA-binding domain of DtxR. Three substitutions occurred within a predicted alpha-helical region with the sequence His98-X_3-Cys102-X_3-His106 that resembles metal-binding motifs in several other proteins and is identified as the putative metal-binding site of DtxR. Second, we targeted the probable metal-coordinating histidine and cysteine residues within the metal-binding sequence of DtxR for oligonucleotide-directed site-specific mutagenesis and isolated six additional DtxR variants.

Gel retardation and DNase I protection assays with DNA fragments containing the *tox* operator were used to examine the DNA-binding activities of wild-type and selected variant DtxR proteins, and a quantitative assay to measure the $^{63}Ni^{2+}$-binding activity of DtxR proteins was developed. Amino acid substitutions within the DNA-binding domain of DtxR destroyed DNA-binding activity but did not alter metal-binding activity. Scatchard analysis revealed that DtxR has a single class of high-affinity $^{63}Ni^{2+}$-binding site with a K_d of 0.98 x 10^{-6} M and maximum binding capacity of approximately 0.8 atom of Ni^{2+} per DtxR monomer, plus a low affinity site(s) for which its K_d(s) was not accurately determined. Since DtxR exists as a dimer (S. Zhang, personal communication), it is presumed that two high-affinity metal-binding sites are formed within the metal-binding domain of DtxR. We concluded that His-106 was essential for the metalloregulatory

function of DtxR, because amino acid substitutions at this position decreased metal-binding activity and destroyed DNA-binding activity of DtxR. In contrast, substitutions for His-98 decreased metal binding activity without abolishing repressor function. Amino acid replacements at Cys-102 residue did not greatly affect metal-binding, but they prevented activation of the DNA-binding function of DtxR. These observations susggested that Cys-102 played a secondary role in metal-binding but was crucial for coupling of metal binding to the DNA-binding activity of DtxR. Based on these experimental results, I propose a model in which the two metal-binding sites in dimeric DtxR are located at the interface between the DtxR monomers, and each metal ion coordinates with both DtxR monomers.

Identification and characterization of the functional domains of diphtheria toxin repressor (DtxR)

by

Zhao-Xi Wang

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology Graduate program of the Uniformed Services University of the Health Sciences in fulfillment of the requirements for the degree of Doctor of Philosophy 1995

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who is the love of my life.

Without her support, I could not pass the hard time in my life.

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INTRODUCTION

Preface

The introduction of this dissertation is divided into five sections. The first section provides a general description of diphtheria caused by *Corynebacterium diphtheriae*. The second section is a discussion of the diphtheria toxin secreted by *C. diphtheriae*. The third section presents an overview of the diphtheria toxin repressor (DtxR). The fourth section provides a description of metalloregulatory proteins. The final section outlines the specific objectives of this dissertation.

I. Overview of Diphtheria Caused by *Corynebacterium diphtheriae*

Diphtheria is an acute, life-threatening bacterial disease caused by *Corynebacterium diphtheriae*, that is spread by respiratory droplets or intimate contact. Before mass immunization against diphtheria toxin became available beginning in the late 1920s, diphtheria was a major cause of death among children. Diphtheria affected up to 10% of the population with mortality rates of 30 to 40% in untreated cases. More than 206,000 cases of diphtheria occurred in the United States in 1921 (McLeod, 1950).

Local infections occur on mucous membranes (primarily in the nasopharynx or larynx) or skin with a characteristic pseudomembrane formation at the site of infection resulting from tissue destruction. Some strains of *C. diphtheriae* produce a soluble exotoxin, diphtheria toxin (DT), which can cause local tissue damage and is also

responsible for the severe systemic manifestations of diphtheria, such as myocarditis and polyneuritis.

The earliest records suggest that diphtheria already existed in the fourth century B.C., and the physician Aretaeus described this disease in A.D. 70 (Fischer, 1991). By pinpointing pseudomembrane, the French doctor Pierre Bretonneau in 1826 established diphtheria as a distinct clinical entity, and he suggested that the disease was caused by a germ and could be transmitted from person to person (Ziporyn, 1988). The causative organism was first observed and described by Klebs in stained smears from diphtheritic membrane in 1883, and its etiologic role was proved one year later by Loeffler when he grew the organism on artificial media and produced a fatal infection closely resembling the human disease in guinea pigs (Pappenheimer and Gill, 1973). Diphtheria toxin was first described in 1888 by Roux and Yersin (McCloskey, 1985). In the early 1890s, von Behring and Kitasato succeeded in immunizing animals with modified toxin and showed that the serum of such immunized animals could protect susceptible animals against the disease (McCloskey, 1985). Facilitated by Schick's discovery of the test to distinguish between susceptible and resistant individuals to diphtheria toxin, von Behring in 1913 demonstrated that serum containing antitoxin to diphtheria toxin could be used for the prevention and treatment of this disease (McCloskey 1985). This led to the development of the toxoid vaccine, the formalin detoxified diphtheria toxin with preserved immunogenicity, by Ramon and Glenny in 1928, which was subsequently used universally for active immunization against diphtheria (Collier, 1990; McCloskey, 1985).

Routine immunization of children in the United States resulted in a marked

reduction of incidence in diphtheria. Only 22 cases were reported from 1980 to 1987, and 5 cases were reported in 1991. In the United States, large local outbreaks of diphtheria occurred in San Antonio, Texas with 196 diphtheria patients in 1970 (McCloskey *et al.*, 1971), and in Seattle, Washington with 1100 cases between 1972 and 1982 (Coyle *et al.*, 1989). Since high rates of immunization are only obtained by school entry, many younger children and older adults are susceptible to diphtheria in the United States. Alcoholism, low socioeconomic status, crowded living conditions, and American Indian ethnic background are significant risk factors in recent diphtheria outbreaks (Chen *et al.*, 1985).

II. Diphtheria Toxin

1. History and biological activities

By using animal models of diphtheria, Loeffler observed in 1884 that the diphtheria bacilli were restricted to the infection sites, although damage to tissues was found systemically. He concluded that this bacterium could produce a soluble poison which could be transported to remote tissues by the blood stream (Pappenheimer and Gill, 1973). In 1888, Roux and Yersin demonstrated that a heat-labile toxin contained in the bacillus-free filtrates from cultures of *C. diphtheriae* could kill guinea pigs (Collier, 1975). Now diphtheria toxin is regarded as one of the major virulence factors of *C. diphtheriae*. This toxin is responsible for most of the local necrotizing lesions and systemic manifestations of diphtheria, including myocarditis, polyneuritis, and other

systemic complications. It causes similar systemic lesions in susceptible experimental animals treated with purified toxin. The fact that immunity to the toxin protected against severe symptoms of diphtheria suggested the clinical importance of this toxin. Neutralization of the toxicity of diphtheria toxin by administrating antitoxin is still the principal treatment for diphtheria in clinical practice. Despite the known biological roles of toxin, toxigenicity and pathogenicity of *C. diphtheriae* are not synonymous. Toxin formation can dramatically increase the severity of infection, but is neither necessary nor sufficient for survival or pathogenicity of this bacterium (Murphy, 1976a). In respiratory diphtheria, toxinogenic strains of *C. diphtheriae* are usually isolated; in cutaneous diphtheria, nontoxinogenic strains are often identified (Holmes, 1994).

2. Mode of action and cellular target

i. Cellular target of diphtheria toxin Although the existence of the diphtheria toxin and its biological actions have been known for a long time, the details of the toxicity of this molecule for cells were uncovered only after the basic framework of the knowledge of protein synthesis was established in the 1950s. The first important discovery in understanding the mode of action of diphtheria toxin was made in 1959 by Strauss and Hendee (Strauss *et al.*, 1959). They detected an early complete inhibition of cellular protein synthesis in intoxicated HeLa cells. This inhibition was also observed in cell-free extracts of any eukaryotic cell type, but it was only found in intact cells from a few animal species. In 1964, Collier showed that the cofactor NAD was required for the inhibition of protein synthesis by diphtheria toxin. Subsequent studies identified the

cellular target of toxin as the eukaryotic polypeptidyl tRNA translocase, the elongation factor 2 (EF-2) (Collier, 1964 & 1967; Bowman, 1970; Gill *et al.*, 1973).

ii. Mechanism of cytotoxicity of diphtheria toxin The mechanism by which diphtheria toxin kills sensitive cells is that the toxin blocks protein synthesis by transferring the ADP-ribosyl moiety of NAD^+ to the diphthamide residue of eukaryotic elongation factor (EF-2) in the cytoplasm:

$$\mathbf{NAD^+ + EF\text{-}2 \rightleftharpoons ADP\text{-}ribosyl\text{-}EF\text{-}2 + nicotinamide + H^+}$$

This reaction is irreversible under physiologic conditions. The ADP-ribosylated EF-2 is unable to support protein synthesis because it lacks the ability to bind to ribosomes, and does not hydrolyze GTP (Raeburn *et al.*, 1968). Since EF-2 contains a unique residue, diphthamide, it is the only protein in eukaryotic cells that can be ADP-ribosylated by diphtheria toxin (Collier, 1967). Cells with mutations that can not produce diphthamide are resistant to diphtheria toxin (Murphy, 1976). Thus, it is commonly accepted that the ADP-ribosylation of EF-2 is the cause of the toxin's lethal effect on the target cell (Murphy, 1976; Pappenheimer, 1977). Nevertheless, one recent report showed that there was a long lag period (6-7 h) between the cessation of protein synthesis and the cytolysis triggered by diphtheria toxin. During this long lag period, extensive DNA fragmentation, which is the distinctive character of programmed cell death, in target cells was observed about 2-3 hours before cytolysis (Chang *et al.*, 1989). Rapid and complete inhibition of

protein synthesis in cultured cells by treatment with the protein synthesis inhibitor cycloheximide or metabolic poisons (NaN_3/2-deoxyglucose) or by incubation of cells in media deficient in essential amino acids did evoke cytolysis, but after a much longer lag period (20-50 h) and without DNA fragmentation. These observations suggest that diphtheria toxin mediated cytolysis is not a simple consequence of translational inhibition. The new hypothesis on the mode of diphtheria toxin action, proposed by Chang *et al*, is that the toxin-induced modification of second messenger substrates in target cells triggers the cell suicide response. Results reported by Morimoto and Bonavida (1992) revealed that ADP-ribosylation of EF-2 by diphtheria toxin is required for apoptosis of the target cell. In contrast, the mutant CRM 197, a mutant diphtheria toxin which does not catalyze ADP-ribosylation of EF-2, did not cause apoptosis in the same diphtheria toxin sensitive cell line. Collier used an attenuated mutant diphtheria toxin DT-E148S in the similar experiments and revealed that this mutant toxin produced the same effect as wild type diphtheria toxin at higher concentration (Kochi and Collier, 1993). These studies demonstrated the importance of protein translation inhibition caused by diphtheria toxin in the target cell death. Details of mechanism of how does the translation inhibition intrigue the suicide program inside the target cells are still not clear and are under intensive study.

iii. Properties of diphtheria toxin Diphtheria toxin can be isolated from culture filtrates of *C. diphtheriae* as a heat-labile protein. At the present time, it has been purified, crystallized, and characterized in many laboratories. Diphtheria toxin is a potent

microbial toxin. In highly susceptible species (rabbit, guinea pig, and monkey), the lethal dose of the pure toxin is about 0.1 μg/kg. Humans are as sensitive as these experimental animals to this toxin on a body weight basis, and 0.1 ng of toxin can produce a visible skin reaction (Collier, 1975 & 1990). From studies with cultured cells, it was estimated that one molecule of diphtheria toxin could kill a susceptible cell (Yamaizumi *et at*, 1978).

Diphtheria toxin is synthesized and released extracellularly as an acidic, globular protein (pI= 4.1) with a molecular weight of 58,342 daltons, which contains a single polypeptide chain of 535 amino acid residues (Collier, 1975). This molecule is a nontoxic precursor containing two disulfide bonds, one of which spans an arginine-rich region that is highly sensitive to trypsin-like proteases (Carroll *et al.*, 1984). Mild trypsinization and reduction convert this nontoxic precursor into a fully toxic one which contains two large fragments; an amino-terminal fragment A (21 kDa) and a carboxyl-terminal fragment B (37 kDa) (Pappenheimer, 1977). The A-fragment carries the intact ADP-ribosyl transferase activity. The B-fragment enables the whole toxin to bind to specific receptors at the susceptible cell surface (Rolf *et al.*, 1990; Middlebrook *et al.*, 1978). The crystallography of diphtheria toxin revealed that this molecule contained three structural domains with distinct functions (Choe *et al.*, 1992; Rolf and Eidels, 1993). The A-fragment harbors the catalytic domain. Fragment B consists two domains, the receptor-binding domain and the translocation domain.

iv. Binding of receptor and translocation The diphtheria toxin receptor is a heparin-binding EGF-like growth factor precursor on the susceptible cell surface (Cieplak *et al.*, 1987). It determines whether a specific cell type is sensitive or resistant to this toxin and is essential for efficient translocation of the A-fragment across the plasma membrane (Naglich *et al.*, 1992; Stenmark *et al.*, 1988). After binding to the plasma membrane receptor, the toxin is internalized in coated pits and delivered to the endosomes (Proia *et al.*, 1981; Morris *et al.*, 1985). The low pH inside the endosome causes conformational changes of the B-fragment, resulting in exposure and insertion of the hydrophobic translocation domain into the endosomal membrane (Sandvig and Olsnes, 1980). By using the energy from the proton gradient and free anion transport, the A-fragment traverses the plasma membrane and enters the cytosol (Olsnes *et al.*, 1988). The translocation of fragment A does not occur and protein synthesis is not inhibited if acidification of endosomes is prevented by NH_4Cl or monensin (Olsnes *et al.*, 1988).

3. Regulation of diphtheria toxin

i. Lysogeny and Toxinogeny The existence of bacteriophages was first reported by d'Herelle in 1918. However, it was not until 1951 that the relationship between toxinogenicity and lysogeny was established by Freeman (Ajl *et al.*, 1970). Subsequent research showed that only those strains of *C. diphtheriae* that were lysogenic for certain tox^+ corynephages produced diphtheria toxin. That is, the nontoxinogenic diphtherial strain $C7(-)^{tox-}$ could be converted to the toxinogenic strain $C7(\beta)^{tox+}$ by lysogenization with the temperate phage β^{tox+} (Singer, 1976). Recombination of the

determinant for diphtheria toxin on corynephage was first demonstrated by Groman and Eaton in 1955 (Groman, 1955). By using doubly infected *C. diphtheriae* C7(-)$^{tox-}$ strain with two closely related phages (γ^{tox-} and β^{tox+}) which differed from each other in host range, they isolated toxinogenic recombinant phages with the γ-phage host range and nontoxinogenic recombinants with the β-phage host range. The *tox* gene was first mapped on the vegetative β-phage genome by Holmes and Barksdale in 1969 (Holmes *et al.*, 1969 & 1970). The structural gene of diphtheria toxin, *tox*, on phage β was discovered by Uchida (1971). Corynephage β is an inducible, temperate phage which has a linear double-stranded DNA genome of about 34.7 kbp with cohesive ends. The *tox* gene is a nonessential gene of phage β, and its expression is not affected by the vegetative replication of the phage or the integration into the host bacterial chromosome (Singer, 1976).

ii. Effect of iron on diphtheria toxin production Although all toxinogenic *C. diphtheriae* strains carry a *tox*$^+$ phage, the production of diphtheria toxin is under the tight control of the environmental iron concentration (Pappenheimer *et al.*, 1936). Toxin is synthesized in high yield only at late logarithmic phase or stationary phase of bacterial growth, when the exogenous iron has become exhausted. Under high-iron conditions, the production of toxin is repressed.

iii. Mechanism of the iron-dependent regulation of diphtheria toxin production Among many hypothesises advanced and tested to explain the iron-

dependent regulation of diphtheria toxin synthesis, the best one was that the iron-dependent binding of regulatory proteins controlled the *tox* expression. Direct experimental evidence supporting this hypothesis came from in vitro diphtheria toxin synthesis from β^{tox+} DNA in cell extracts of *Escherichia coli* (Murphy *et al*, 1974). Diphtheria toxin was produced from β^{tox+} DNA in *E. coli* extracts, but a high concentration of iron failed to inhibit expression of the *tox* gene in this *E. coli* system. On the contrary, a similar cell extract system from the nontoxinogenic strain *C. diphtheriae* C7(-)$^{tox-}$ programmed with β^{tox+}DNA showed complete inhibition of toxin production by iron. When a small amount of supernatant of cell extract from nontoxinogenic C7(-)$^{tox-}$ strain was added to the *E. coli* system, iron-dependent repression of *tox* expression was observed. The regulatory factor in C7(-)$^{tox-}$ extracts was further partially purified, and it appeared to be an iron-containing protein which exhibited an iron-dependent binding to [^{32}P]DNA when the protein was immobilized on nitrocellulose filters (Murphy *et al.*, 1976). Therefore, iron appears to act as a corepressor in *C. diphtheriae* in controlling diphtheria toxin production. In 1976, the current model for regulation of the *tox* gene was proposed by Murphy *et al*, i.e. that a chromosomally encoded repressor of *C. diphtheriae* regulates the expression of the *tox* gene on corynephage in response to environmental iron concentrations (Murphy *et al.*, 1976) (Fig. 1). Based on this model, two classes of mutants derived from toxinogenic strains of *C. diphtheriae* which were relatively insensitive to iron repression were anticipated.

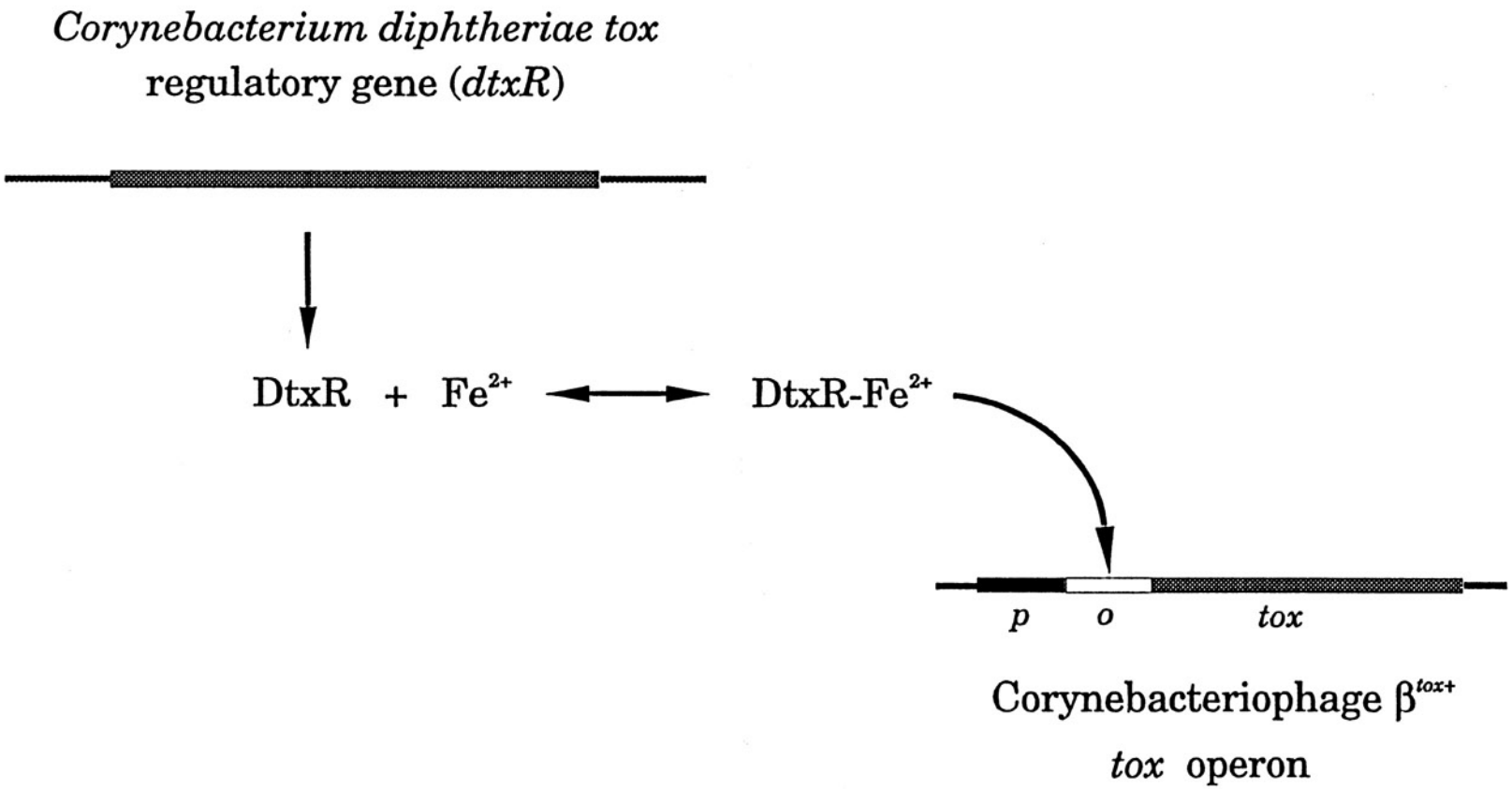


Figure 1. Model of corynebacteriophage β *tox* gene regulation by *C. diphtheriae*. A repressor is encoded on the chromosome of *C. diphtheriae* which in the presense of iron binds to the operator of the *tox* gene. Under conditions of iron starvation the process can be reversed.

Mutations in repressor gene The first class of mutants was expected to contain mutations in the putative repressor gene located on the chromosome of *C. diphtheriae*. The variant repressor produced from this type of mutant would not bind to the *tox* promoter/operator, resulting in high expression of the *tox* gene under high-iron conditions. *C. diphtheriae* C7hm723(β), a derivative of *C. diphtheriae* C7, was a mutant of this type isolated by Kanei *et al* that produced toxin constitutively in the presence of excess iron (Kanei *et al.*, 1977). The β^{tox+} phage isolated from this mutant strain could convert a nontoxinogenic C7(-) strain into a toxinogenic strain that was fully sensitive to iron repression. After the repressor gene had been discovered, the mutant repressor gene for C7hm723 was also cloned and sequenced. It contained a single-base change causing the replacement of arginine-47 by histidine-47, and it failed to bind to a DNA fragment containing the *tox* promoter/operator region in in vitro DNA-binding assays (Schmitt and Holmes, 1991b & 1993; Boyd *et al.*, 1992).

Mutations in the operator of the *tox* gene The second class of mutants was expected to be located in the putative *tox* operator of corynephage β. This type was the operator-constitutive (O^c) mutants analogous to the *lac* O^c mutants of *E. coli*. A β-phage mutant $\beta^{tox+,\ ctI}$ isolated by Murphy *et al* (1976b), was partially insensitive to iron mediated repression, and it was *cis* dominant in the double lysogen C7($\beta^{crm+,\ 45}/\beta^{tox+,\ ctI}$). The mutation in $\beta^{tox+,\ ctI}$ was presumed to be located in the *tox* operator. The constitutive *tox* promoter/operator mutants $\beta^{tox\text{-}201}$ and $\beta^{tox\text{-}202}$, isolated by Welkos and Holmes, contained G to A base changes at position -47 and -48 within the putative -10 sequence

of the *tox* promoter, respectively (Welkos and Holmes, 1981 and Krafft *et al.*, 1992). These mutants had elevated activities of the *tox* promoter and partial resistance of the operator to iron-dependent repression.

III. Diphtheria Toxin Repressor (DtxR)

1. Discovery of diphtheria toxin repressor and biological activities

Isolation of two classes of iron insensitive mutants from *C. diphtheriae* strain C7(β)$^{tox+}$ strongly suggested that the repressor acted at the transcription level in iron-dependent regulation of the *tox* gene. The kinetics of inhibition of diphtheria toxin production from C7(β^{tox+}) by iron and by rifampin, an inhibitor of transcription initiation, were almost identical (Murphy, 1978). These data eliminated the possibility that the repressor acted at the translation level . Further studies showed that there was a large amount of *tox* mRNA in the pool of [^{3}H]RNA extracted from iron-limited C7(β^{tox+}), but not from C7(β^{tox+}) before the onset of toxin production or from nonlysogenic C7(-). Quantitation of *tox* mRNA by dot blot hybridization from *C. diphtheriae* in the presence or absence of iron gave the first direct evidence that iron regulated diphtheria toxin expression at the level of transcription (Kaczorek *et al.*, 1985). To facilitate further studies on the mechanism of iron dependent regulation, the efficiency of the *tox* promoter was compared in *C. diphtheriae* and *E. coli* backgrounds by comparison of the β-galactosidase activities directed by this promoter. Results indicated that the *tox* promoter had about 10% of the efficiency of the *lac* promoter, and had about 40 times higher

efficiency of transcription in *C. diphtheriae* than in *E. coli* (Kaczorek *et al.*, 1985). Nuclease S1 mapping of mRNA and primer extension experiments revealed there were the same 5′ terminal nucleotide of *tox* mRNA in *C. diphtheriae* and *E. coli*. These observations demonstrated the *tox* promoter had the same transcriptional initiation site both in *C. diphtheriae* and *E. coli*. Nucleotide sequence analysis revealed that the *tox* promoter carried a putative -35 and two -10 sequences closely related to the *E. coli* consensus sequences (Kaczorek *et al.*, 1985; Leong *et al*,. 1985). By using oligonucleotide-directed mutagenesis, Boyd *et al* showed that the putative -10 region of the *tox* promoter at position -50 from the translational initiation codon was favored during the expression from the *tox* promoter (Boyd *et al.*, 1988). From in vitro protein-DNA binding experiments, including gel mobility shift assays and DNase I protection experiments, a protein in crude extracts from *C. diphtheriae* was found to bind in an iron-dependent manner to a palindromic motif (containing a 9 base-pair inverted repeat sequence) in the *tox* promoter overlapping the -10 region, which was designated as the putative *tox* operator (Fourel *et al.*, 1989). Binding of the factor was presumed to prevent the interaction between the transcriptional initiation machinery and the *tox* promoter. All of these findings were consistent with the proposed model of iron-dependent *tox* regulation.

In order to find the iron-dependent regulatory factor, reporter systems containing *tox-lacZ* operator or gene fusions, in which *lacZ* expression was under control of the *tox* promoter-operator region, were constructed and introduced into *E. coli*. The expression of *lacZ* from these reporter constructs was not affected by the *E. coli* iron-dependent

regulatory protein Fur (Boyd and Murphy, 1990). Genomic libraries of DNA from nontoxinogenic, nonlysogenic *C. diphtheriae* were screened for repression of *lacZ* expression from these reporters on high-iron medium. By using this strategy, the gene of the diphtheria *tox* iron-dependent regulatory element, or diphtheria toxin repressor named (DtxR), was cloned and sequenced (Boyd *et al.*, 1990; Schmitt and Holmes, 1991a). The deduced amino acid sequence contains 226 residues with a predicted molecular mass 25,316 daltons. Computer searches of Genebank at the nucleotide and amino acid levels revealed that *dtxR* was unique, but it had a low level of homology with the *fur* gene of *E. coli*. DtxR protein encoded by the recombinant *dtxR* allele in *E. coli* was purified, and rabbit antiserum against DtxR was prepared. A partial N-terminal polypeptide sequence of this protein was determined by sequential Edman degradation, and it corresponded exactly with the N-terminal sequence of DtxR as deduced from the nucleic acid sequence (Tao *et al.*, 1992; Schmitt *et al.*, 1992).

In the *E. coli* system, DtxR acted as a negative controlling element for expression of the *tox*P/O-*lacZ* fusion in an iron-dependent manner (Boyd and Murphy, 1990; Schmitt and Holmes, 1991a). When the cloned, wild type *dtxR* gene was introduced into the mutant strain *C. diphtheriae* C7(β)hm723, which produced diphtheria toxin constitutively under high-iron conditions, it restored the repressibility of toxin production by iron (Schmitt and Holmes, 1991a). Further, it was shown that the system of the high-affinity iron-chelating siderophore of the iron uptake system in *C. diphtheriae* was coordinately regulated with diphtheria toxin by DtxR (Tai *et al.*, 1990). In a recent report, a partially sequenced gene, which was under the control of a DtxR-regulated promoter IRP1, was

homologous with a family of periplasmic proteins involved in iron transport in gram-negative bacteria and with the ferrichrome receptor, FhuD, of *Bacillus subtilis* (Schmitt and Holmes, 1994). These findings suggested that DtxR also controls expression of the high-affinity iron uptake system in *C. diphtheriae*.

2. Functions associated with DtxR

The proposed mechanism of action for DtxR is similar to other metal-dependent regulatory proteins. It was presumed that metal ions could activate DtxR to bind to the *tox* operator region and inhibit the interaction of the transcription machinery with the *tox* promoter. There are several predicted functions associated with DtxR:

i. DNA-binding activity Direct evidence for DNA-binding of DtxR came from in vitro DNA-binding assays, including gel mobility shift assays and footprint assays, by using highly purified, recombinant DtxR from *E. coli* with DNA fragments carrying the *tox* operator sequence (Schmitt *et al*, 1992). DNase I protection experiments demonstrated that purified DtxR in the presence of Fe^{2+}, as well as certain other divalent cations, protected a 30 bp region of the *tox* operator (Schmitt and Holmes, 1993; Tao and Murphy, 1992a and 1992b). This protected region, covering the putative -10 region of the *tox* promoter and containing a 9 bp dyad symmetric sequence (Schmitt *et al.*, 1992; Tao, *et al.*, 1992), was almost identical to the sequence protected by the factor from the crude extract of *C. diphtheriae* described by Fourel (Fourel *et al*, 1989). A single protein with molecular weight 28 kDa detected by anti-DtxR antiserum in crude extracts of both

recombinant *E. coli* and *C. diphtheriae* strain C7(-) suggested that DtxR was identical to the factor described by Fourel (Tao *et al*, 1992). Analysis of two additional DtxR-regulated promoters from *C. diphtheriae* as well as the homologous *des* promoter (iron regulated) from *Streptomyces pilosus* (Schmitt *et al.*, 1994; Günter *et al.*, 1993) (Fig. 2) identified a conserved 19-bp palindromic DNA core region within the recognition sequences for DtxR-regulated promoters. Like the Fur protein, an iron-dependent repressor in gram-negative bacteria, DtxR seems to control a family of promoters which contains a so called DtxR-binding box, and it seems to serve as a counterpart of Fur in gram-positive bacteria (Schmitt and Holmes, 1994).

The variant DtxR purified from the C7hm723(-) strain of *C. diphtheriae*, containing a single amino acid substitution of histidine for arginine at position 47, failed to regulate the expression of β-galactosidase from the *toxP/O-lacZ* fusion gene (Schmitt and Holmes, 1991b; Boyd *et al*, 1992). This variant DtxR also failed to protect the *tox* operator from DNase I digestion in the presence of 1.5 μM Co^{2+}, but wild type DtxR exhibited full protection at this concentration of Co^{2+} (Schmitt *et al.*, 1991b & 1993). These findings were initially interpreted by postulating that the decreased repressor activity of DtxR-R47H was caused by a decrease in its binding affinity for divalent cations. Results described in this dissertation led to a revision of this original explanation for the decreased repressor activity of DtxR-R47H.

The DNA fragments containing the *tox* operator from the constitutively tox^+ β-phage mutant $\beta^{tox\text{-}201}$ were also subjected to DNase I protection assays and showed a very weak interaction between wild type DtxR and the *tox-201* promoter/operator sequence

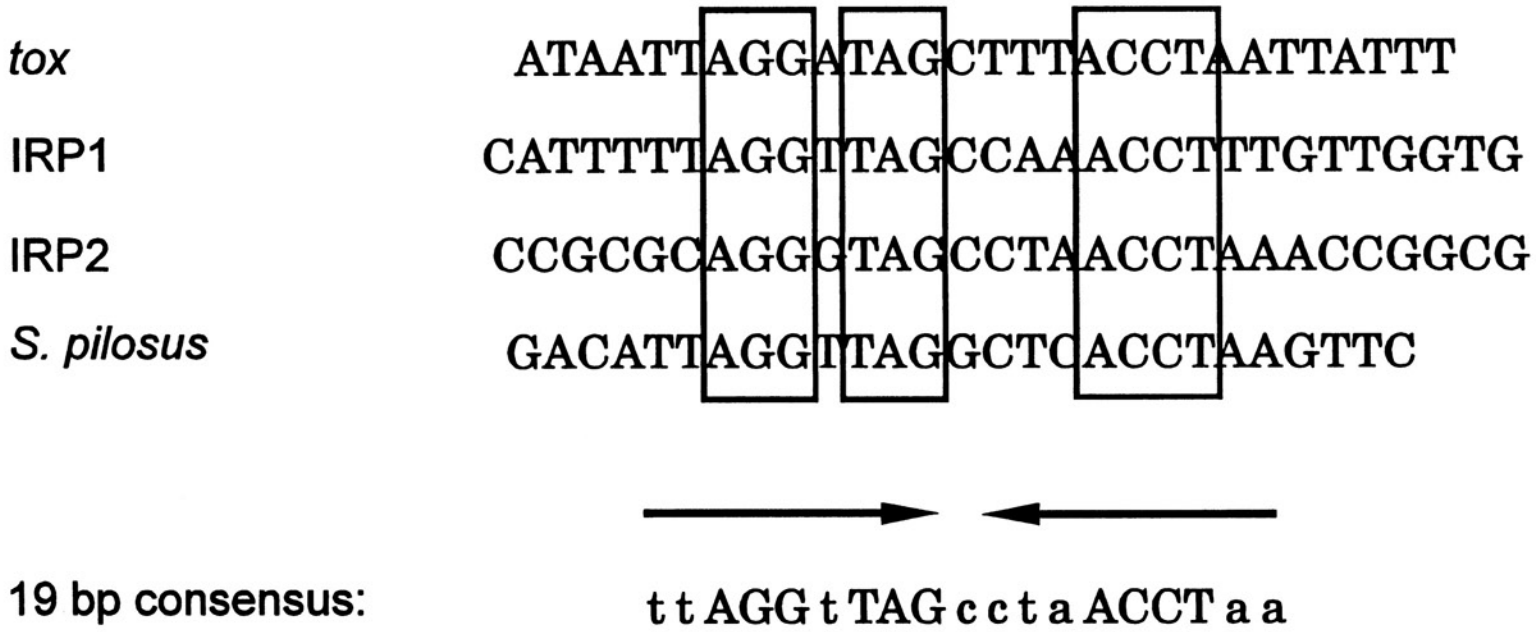


Figure 2. "DNA sequence alignment of the DtxR-binding sites from promoters *tox*, IRP1, IRP2, and the putative DtxR-binding site from *Streptomyces pilosus*. Boxed sequences indicate bases that are conserved in all four operators. A 19-bp consensus sequence which was derived from a comparison of the four sequences is shown. Within the 19 bp consensus sequence, inverted repeats are indicated by arrows, nucleotides that are strictly conserved are shown in capital letters, and nucleotices that are less well conserved are shown in lower case letters." (Schmitt and Holmes, 1994).

(Schmitt and Holmes, 1992). Thus, the single G•C to A•T base-pair substitution at position -47 in the *tox-201* allele is sufficient to interfere dramatically with binding of DtxR to the *tox* operator. This finding confirmed and extended previous analysis of the *tox* regulatory region and showed that the nucleotide at position -47 was important for sequence-specific binding of DtxR to the *tox* operator.

ii. Metal-binding activity When the present studies were begun, no experimental evidence directly showed the metal-binding activity of DtxR. Indirect evidence came from in vitro experiments showing that the binding of DtxR to the *tox* operator required divalent heavy metal cations (Schmitt *et al.*, 1992 & 1993; Tao *et al.*, 1993). Active divalent cations included Fe^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+}, but Cu^{2+} failed to activate DtxR in protecting the *tox* operator. According to the model of another well characterized metal-dependent regulatory protein, MerR, it was presumed that binding of divalent cation to DtxR caused an allosteric change in protein which enables DtxR to bind to the *tox* operator.

iii. Dimer formation The presumption that the functional DtxR is a dimer or multimer form was supported by the results of in vitro DNA-binding assays. The specific DtxR-binding region of the *tox* operator contained a 9-bp inverted repeat, and the specific contacts between DtxR and the deoxyribose-phosphate backbone revealed by hydroxyl radical protection experiments were located in a symmetrical manner about the dyad axis of the *tox* operator (Schmitt and Holmes, 1993). Direct evidence came from protein

crosslinking studies and HPLC chromatography which demonstrated that DtxR exists as a dimer both with and without divalent cations (Zhang *et al.*, personal communication).

IV. Metalloregulatory Proteins

1. Definition

Metalloregulatory proteins can transduce transition metal signals into changes in gene expression (O'Halloran, 1993). The transition metal signals changes in the intra- or extracellular concentrations of metal ions or metal-ligand complexes. At the physiological level, the metalloregulatory proteins serve both sensory roles and regulatory roles in the genetic switching mechanism. Both in vivo and in vitro experiments demonstrated that in response to changes in the concentration of ferrous iron, DtxR functions as a genetic switch for the *tox* gene and genes of the iron uptake system in *C. diphtheriae*. Accordingly, DtxR belongs to the metalloregulatory protein family, and it shares many common features with other well characterized proteins in this protein family such as Fur and MerR (Table 1).

2. Common features of metalloregulatory proteins

i. Separated metal-binding domain and DNA-binding domain The metalloregulatory proteins usually contain high-affinity metal centers with a variety of coordination geometries, and they can specifically recognize the metal ion from the intracellular pool of low molecular weight metal complexes. The DNA-binding domains

a. Parentheses indicate that aspects of the coordination chemistry have yet to be established.

b. Bold indicates metal-binding sites that have been directly established with the use of physical methods. C, Cys; D, Asp; H, His; I, Ile; K, Lys; P pro. NA, not availible.

Table 1. Metalloregulatory Proteins (O'Halloran, 1993)

Protein	Metal Ions[a]	Metal-Binding Sequence[b]
ACE1	Cu cluster	$CxxCx_3Hx_4CxxHx_{17}CxHC_{14}CxCx_{16}CxCx_5CxCH$
ARS	(As)	NA
CopR-CopS	(Cu)	NA
DtxR	(Fe)	$Hx_{18}HxxxCxxxHx_{94}Hx_4Hx_{12}H$
Fur	(Fe)	$HHx_{37}Hx_{14}HHHxHxxCxxCx_{21}Hx_6Hx_6HCx_4Cx_4HxH$
IRE-BP	Fe	$\mathbf{Cx_{65}CxxC}$
MerR	Hg 1/dimer	$\mathbf{Cx_{34}Cx_8C}$
PcoR-PcoS	(Cu)	NA
HAP1	(heme)	$\mathbf{K_{CP}I_{DH}}$

of these proteins are usually located separately from the metal-binding domains. The metal signal usually transduces from the metal-binding domain to the DNA-binding domain on these genetic switches (O'Halloran, 1993).

ii. The allosteric switching functions Binding of metal ions to metalloregulatory proteins can cause allosteric changes which enable these proteins to bind to DNA fragments carrying the corresponding regulatory elements. The result of allosteric switching is either a positive regulation which turns on gene expression, or a negative one which shuts down the gene expression (O'Halloran).

3. Structure-function analysis of metalloregulatory proteins

Metalloregulation may be an important aspect of simple switches or an integral component of complex signal transduction networks. Studies of metalloregulatory proteins can provide information about the molecular basis of metal ion recognition and the intracellular pool of metal complexes, and descriptions of their functions at the molecular level can also provide the basis for models of metalloregulation in cell biology and metal ion toxicology. Furthermore, these studies can provide clues for the design of inorganic pharmaceuticals and other agents that will target metal-controlled pathways. The way to understand the mechanism of regulation, metal specificity, and sensitivity of switching devices is to establish the structure-function relationships of these metalloregulatory proteins. The following sections describe two well characterized metalloregulatory proteins.

i. MerR protein, a mercury-dependent regulator of eubacteria

Bacterial resistance to the toxic effects of the heavy metal mercury ion Hg^{2+} is common in both gram-negative and gram-positive bacteria (Helmann *et al*, 1989). Mercury resistance is mediated by the gene products of the *mer* operon. Genes *merT* and *merP* from this operon encode membrane and periplasmic proteins, respectively, which are involved in Hg^{2+} uptake, and the *merA* gene encodes a mercuric ion reductase that detoxifies the internalized Hg^{2+} ion into nontoxic Hg^0. The transcriptional activity of the *mer* operon is controlled by one of the operon gene products, the regulatory protein MerR (Foster, 1987). MerR can activate transcription from the *mer* promoter in the presence of Hg^{2+} and repress transcription from this promoter in the absence of Hg^{2+} (Lund *et al.*, 1986). In addition, MerR negatively regulates its own synthesis, both in the presence and absence of Hg^{2+}, from an overlapping but divergently oriented promoter.

In vitro $^{203}Hg^{2+}$-binding studies with purified MerR indicate that cysteine residues within the polypeptide sequence of MerR are potential ligands for Hg^{2+}, and only one Hg^{2+} molecule binds to a MerR dimer (Shewchuk *et al.*, 1989a and 1989b). Genetic data from random mutagenesis of the *merR* gene with hydroxylamine demonstrate that mutations which cause defective regulation of MerR are clustered in two functional domains, the DNA-binding domain (defective in both repression and activation) and the Hg^{2+}-binding domain (defective in activation but not in repression) (Ross *et al.*, 1989). Site-directed mutagenesis of the cysteine residues revealed that three of four cysteine residues per monomer of MerR are required for Hg^{2+}-binding (Ross *et al*, 1989; Helmann *et al.*, 1990) . Subsequently, heterodimer complementation experiments indicated that

Cys-79 in one subunit and Cys-114 and Cys-123 in the second subunit are necessary and sufficient for high-affinity Hg^{2+}-binding in an asymmetric, subunit bridging coordination complex (Shewchuk *et al*, 1989a & 1989b; Helmann *et al.*, 1990).

Both MerR and Hg^{2+}-MerR can bind to the *mer* operon and bend DNA, but the prior acts as repressor, and the latter acts as activator, respectively. Binding of Hg^{2+} to MerR not only causes an allosteric change in the protein, but it also induces a localized distortion of the DNA. This distorted DNA bound by Hg^{2+}-MerR in the activated state is underwound at least 30° more than undistorted DNA bound by MerR without Hg^{2+} in the repressed state. This signal-responsive conformational change of the DNA makes it a better template for RNA polymerase (Ansari *et al.*, 1992; Lee *et al.*, 1993).

ii. Fur protein, an iron-dependent repressor in *Escherichia coli*

Iron is an extremely important element for biological systems, but it is also a potentially toxic element because it can catalyze the formation of reactive hydroxyl radicals which can damage all cellular constituents (Foster and Hall, 1992; Halliwell, 1988). Thus, it is necessary to tightly regulate the intracellular concentration of iron. Under conditions of iron starvation, *Escherichia coli* derepresses a number of genes that code for multiple high-affinity iron-uptake pathways (Bagg and Neilands, 1987b), as well as the genes for the colicin I receptor (Griggs and Konishy, 1989) and Shiga-like toxin (Calderwood and Mekalanos, 1987). All these genes are repressed under high-iron conditions by the product of the *fur* gene (ferric up regulation) (Hantke, 1981). Fur-like regulatory systems are ubiquitous in gram-negative bacteria, such as in *Vibrio cholerae*

(Litwin *et al.*, 1992), *Yersinia pestis* (Staggs and Perry, 1991), and *Pseudomonas aeruginosa* (Prince and Vasil, 1992). Fur acts not only as a metal-responsive repressor but also as a positive activator of acid-induced stress proteins (Foster and Holly, 1991), and it affects the synthesis of dicarboxylic acids, manganese resistance, and expression of the manganese form of superoxide dismutase (Neiderhoffer *et al.*, 1990). When it acts as a repressor, complexes of Fur with Fe^{2+} or several other divalent cations (including Mn^{2+}, Co^{2+}, Cd^{2+}, and Cu^{2+}), bind to a 17-bp consensus DNA recognition sequence (the iron box) with dyad symmetry located upstream from iron-regulated genes (Bagg and Neilands, 1987a; de Lorenzo *et al.*, 1987). Little is known about the structure of the Fur protein, the stoichiometry of the complex, or the metal-binding domain of Fur. Nuclear magnetic resonance (NMR) studies indicated that two or three histidine residues located at the carboxyl terminal end of Fur are involved in metal-binding (Saito *et al.*, 1991). Protease-susceptibility studies demonstrated that the Fur protein undergoes a conformational change upon binding of divalent cations (Coy and Neilands, 1991).

V. Specific Aims

1. Summary and the remaining problems

Fur and related proteins are ubiquitous iron-dependent repressors in gram-negative bacteria, and DtxR may be the prototype for a similar family of iron-dependent repressors in gram-positive bacteria. Fur and DtxR share the same mechanism of iron-dependent regulation (Fig. 1, p 11). When the coorepressor Fe^{2+} is present, functional repressor is

formed by binding of corepressor to aporepressor. The functional repressor can bind to specific operator sequences on DNA, resulting in inhibition of transcription from the regulated promoters. DtxR and Fur have a low degree of homology, recognize significantly different consensus sequences, and cannot substitute for one another in vivo (Schmitt and Holmes, 1991a).

Although we have learned a lot about the functions of DtxR, the details of its structure-function relationships are still not clear. Questions that remain to be answered include:

1. Which amino acid residues are required to form the DNA-binding domain, the metal-binding domain, and the dimer-association domain?
2. What are the structural features of these functional domains?
3. How does metal-binding result in activation of the DNA-binding domain?

2. Specific aims of research project

The major objective of my research project was to characterize the functional domains of DtxR, mainly focusing on the metal-binding domain and the DNA-binding domain. The five specific aims designed to achieve this objective were as follows.

1. Isolation and characterization of DtxR variants defective in repressor activity. In vitro random mutagenesis was used to obtain mutations at various sites of the *dtxR* target gene, and the reaction was controlled so that approximately only one amino acid substitution occurred per DtxR polypeptide encoded by the mutagenized gene.

The mutant *dtxR* alleles were generated by mutagenesis with sodium bisulfite. Then these mutant alleles were screened and characterized for their repressor-deficient phenotypes in a test strain of *E. coli*.

2. Prediction of the DNA-binding domain and the metal-binding domain of DtxR. Sequence analysis of mutant *dtxR* alleles was performed, and the distribution of amino acid substitutions along the polypeptide sequence of mutant forms of DtxR was analyzed. By comparing these findings with the polypeptide sequences of other well known DNA-binding proteins and metal-binding proteins, the putative DNA-binding and metal-binding domains of DtxR were deduced.

3. Construction and characterization of site-specific DtxR variants. According to the predictions from specific aim 2, particular residues in the putative metal-binding domain were selected as targets for modification by oligonucleotide-directed site-specific mutagenesis. The mutant alleles constructed by site-specific mutagenesis were then tested for their repressor phenotypes.

4. Development of a metal-binding assay for DtxR. Since no method was available for measuring the metal-binding activity of DtxR when these studies were begun, an assay for this purpose was developed.

5. Characterization of DtxR variants by in vitro experiments. Several DtxR variants

with amino acid substitutions within the DNA-binding domain or the metal-binding domain were purified and characterized by DNA-binding assays and metal-binding assays performed in vitro.

MATERIALS AND METHODS

1. Plasmids and Bacterial Strains

E. coli K-12 strains CJ236, DH5α, MC1009, and XL-1 blue (Bethesda Research Laboratories, Gaithersburg, Md.) were used for all experiments. Stock cultures were stored at -70° C in Luria Broth medium with 20% glycerol. The strains and plasmids used in this dissertation are listed in Table 2 and Table 3.

2. Media, Growth Conditions, Enzymes, and Reagents

Media and growth conditions *E. coli* strains were routinely cultured in Luria Broth (LB) medium or on LB agar medium (Maniatis *et al*, 1989) at 37°C. LB medium contains about 30 μM iron and is considered to be a high-iron medium. The following supplements were added as needed: ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). LB medium was made low-iron by the addition of an iron chelator, ethelenediamine-di-*o*-hydroxyphenyl acetic acid (EDDA) (Sigma Chemical Co., St. Louis, Mo.), at 500 μg/ml; and LB agar medium was made iron-deficient by adding EDDA at 40 μg/ml (Schmitt *et al.*, 1991b). EDDA was deferrated by the method of Rogers (1973) prior to use as an iron chelator.

Table 2. *E. coli* Strains used in this study

STRAINS	RELEVANT CHARACTERISTICS	SOURCE[a]
CJ236	*dut1 ung1 thi-1 relA1* F′ [pCJ105 (Cm^r)]	BRL
DH5*α*	F^- *supE44 ΔlacU169 (ϕ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	BRL
MC1009	F^- *Δ(lacIPOZY) chi74 Δ(ara-leu)7697 galK gal U recA rpsL* λ^-	BRL
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1* lac^- F′ [$proAB^+$ $lacI^q$ *lacZΔM15 Tn10* (Tet^r)]	BRL

[a] BRL, Bethesda Research Laboratories.

Table 3. Recombinant plasmids used in this study

PLASMIDS	RELEVANT CHARACTERISTICS	REFERENCE
pBluescriptSK	*lacZ* promoter for blue/white color selection or fusion protein induction with IPTG and cloning vector (Amp^r)	Stratagene
pMS298	pBluescriptKS carrying 1.4-kb *Pvu*II insert containing the *dtxR*$^+$ allele (Amp^r)	Schmitt *et al*, 1991a
pSKdtxR	0.8-kb *Nco*I-*Nar*I insert from pMS298 carrying the *dtxR*$^+$ allele (without its native promoter) under *lacZ* promoter control in pBluescriptSK vector (Amp^r)	This study
pDtxR-7	pBluescriptKS carrying 1.1-kb *Bgl*II/*Eco*RI insert containing modified *dtxR* allele (high production of DtxR) (Amp^r)	Schmitt *et al*, 1993
pSKlac$^-$	pBluescriptSK vector with *lacZ* deletion (*Pvu*II-*Pvu*II) (Amp^r)	This study
pCMZ100	*tox-lacZ* translational fusion in *E. coli-C. diphtheriae* shuttle vector pCM2.6 (Cm^r)	Schmitt *et al*, 1991b
pDSK29	5-K fragment carrying *dtxR*$^+$ allele in RSF1010-derived vector (low copy number) (Kan^r)	Schmitt *et al*, 1991a
pPOB	pUC19 derivative carrying 316-bp *Eco*RI/*Hae*III insert containing *tox* promoter/operator region (Am^r)	Schmitt *et al*, 1992
pGP1-2	pACYC184 derivative carrying temperature-inducible T7 RNA polymerase (Kan^r)	Schmitt *et al*, 1991a

Enzymes Restriction enzymes, RNase, DNase I, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 polynucleotide kinase, and T4 ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Stratagene (La Jolla, Calif.), and New England Biolabs (Beverly, Ma). T7 DNA polymerase (sequenase 2.0) for sequencing was from United States Biochemical, Cleveland, Ohio.

Chemicals Bacterial growth supplements, including antibiotics, X-gal, *o*-nitrophenyl-β-D-galactoside (ONPG), isopropyl-β-D-thiogalactopyranoside (IPTG), EDDA, and mutagens, including sodium bisulfite and hydroquinone, were obtained from Sigma Chemical Co., St. Louis, Mo. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were from United States Biochemical, Cleveland, Ohio. The Ni^{2+}-NTA-agarose resin was purchased from Qiagen, Chatsworth, Calif. Isotopes, [α-^{35}S]dATP, [α-^{32}P]dCTP, and $^{63}Ni^{2+}$ (900 Ci/mol) were purchased from Amersham Life Science, Arlingon Heights, Ill.

3. Recombinant DNA Techniques

Plasmid DNA was purified from *E. coli* K-12 strains by alkaline lysis (Birnboim *et al.*, 1979) or by Qiagen preparation (Qiagen Inc., Chatsworth, Calif.). DNA fragments used for subcloning were generated from plasmid DNA digestion with restriction endonucleases, separated by agarose gel electrophoresis, and eluted from the agarose gel matrix with Geneclean system (Bio101, La Jolla, Calif.). T4 ligase was used to ligate

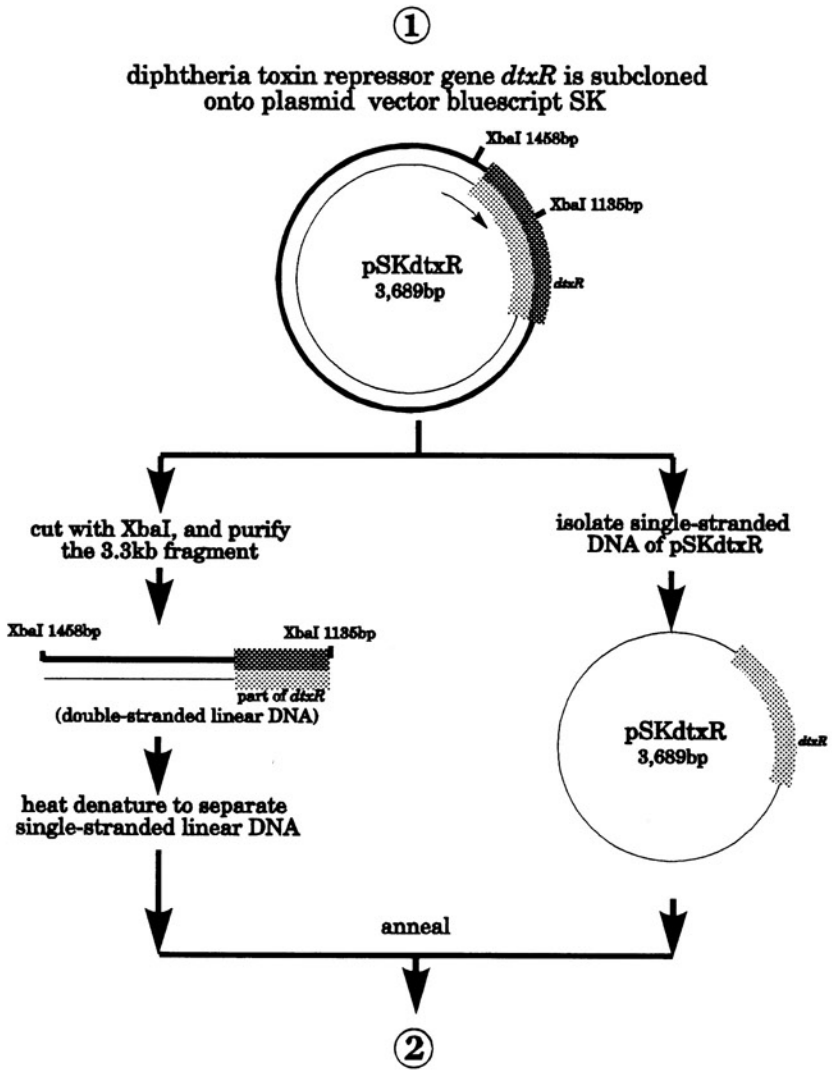
purified DNA fragments into a new recombinant plasmid, and the newly constructed plasmid was subsequently transformed into *E. coli* K-12 made competent for uptake of DNA by treatment with calcium chloride and heat shock (Maniatis *et al.*, 1989).

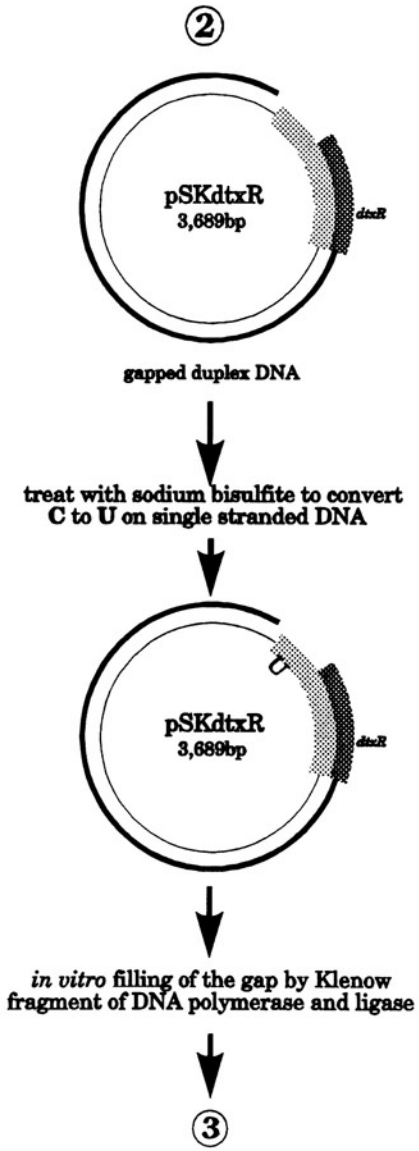
4. Random Mutagenesis with Sodium Bisulfite

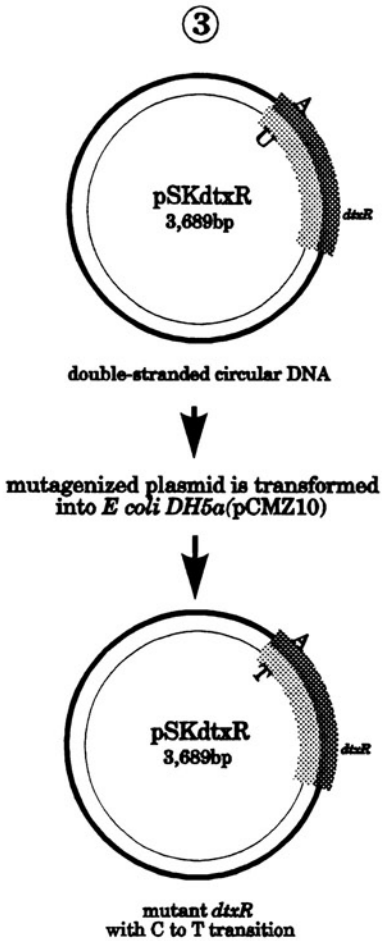
The mutagen sodium bisulfite can specifically deaminate cytosine to uracil within a single-stranded region of DNA. When the mutagenized single-stranded DNA is copied by DNA polymerase, adenine in the newly synthesized DNA strand pairs with the uracil, causing a C•G to T•A transition mutation when the mutagenized DNA replicates in *E. coli* K-12. We performed bisulfite mutagenesis as described by Pine and Huang (Pine and Huang, 1987). The entire process requires the following three steps outlined in Figure 3.

i. Construction of the gapped duplex DNA containing the single-stranded target region. The phagemids pBluescript SK(+) and SK(-), which can exist in both double- and single-stranded forms, were used as vectors in this mutagenesis (described in Bio-Rad Muta-Gene manual, Richmond, Calif.). The wild type *dtxR* allele (*Nco*I/*Nar*I) was subcloned into the multi-cloning site of these vectors, and the newly constructed plasmids, named pSKdtxR(+) and pSKdtxR(-), were then transformed into *E. coli* K-12 strain XL1-blue. When these transformants were superinfected with the helper phage R408, proteins encoded by the helper phage acted at the single stranded origin *f1* of the

Figure 3. Construction of *dtxR* mutations by random mutagenesis with sodium bisulfite. The example shown is for mutagenesis of the 5′ third of the *dtxR* gene located in the 323 bp *Xba*I fragment (see text).


①
diphtheria toxin repressor gene *dtxR* is subcloned onto plasmid vector bluescript SK
XbaI 1458bp
XbaI 1135bp
pSKdtxR
3,689bp
dtxR
cut with XbaI, and purify the 3.3kb fragment
isolate single-stranded DNA of pSKdtxR
XbaI 1458bp
XbaI 1135bp
part of dtxR
(double-stranded linear DNA)
heat denature to separate single-stranded linear DNA
anneal
②


②
pSKdtxR
3,689bp
dtxR
gapped duplex DNA
treat with sodium bisulfite to convert C to U on single stranded DNA
U
in vitro filling of the gap by Klenow fragment of DNA polymerase and ligase
③


③
U
pSKdtxR
3,689bp
dtxR
double-stranded circular DNA
mutagenized plasmid is transformed into *E coli DH5a*(pCMZ10)
T
mutant *dtxR* with C to T transition

phagemids and caused the phagemid DNAs to be replicated, packaged, and extruded fromthe cell as single-stranded phages. By using this method, the single-stranded, full length, genomic DNAs from pSKdtxR(+) or pSKdtxR(-), which contained the sense and antisense strand of *dtxR,* respectively, were isolated from these transformants (Maniatis *et al.*, 1989). In addition, the double-stranded plasmids were isolated from these transformants by using standard methods for plasmid preparation. By using selected restriction endonucleases (*Xba*I, *Pst*I/*Sph*I, and *Sph*I/*Sal*I), three different pairs of linear, double-stranded DNA fragments were generated from genomic DNAs of pSKdtxR(+) and pSKdtxR(-). Six different gapped duplex DNA molecules were constructed by annealing 2 μg of each linear, double-stranded DNA fragment with 1 μg of single-stranded, genomic DNA (sense or antisense, as appropriate) in annealing buffer (3 mM sodium citrate, 30 mM NaCl, and 10 mM $MgCl_2$) (Table 4, p 72). The annealing mixture with final total DNA concentration of 15 μg/ml was heated to 90°C for 2 min, cooled quickly to 80°C and held for 3 min, cooled quickly to 70°C and held for 10 min, cooled quickly to 65°C and held for 50 min, and then slowly cooled to 25°C. The formation of gapped duplex molecules was analyzed by electrophoresis on 1% agarose gels in TAE buffer (Fig. 4). On each gapped duplex molecule, one third of the *dtxR* gene (corresponding with the N-terminal, central, or C-terminal region, approximately 200-300 bp each) was exposed in single-stranded form as the target for mutagenesis, and the rest of *dtxR* gene and vector were protected from mutagenesis by virtue of their double-stranded structure.

Figure 4. Effect of linear DNA to single-stranded DNA ratio in formation of the gapped duplex molecule. The gapped duplex DNA was examined by electrophoresis on a 1.0% agarose gel. Lane 1 is the reaction mixture before annealing (ds/ss ratio of 4:1). Lanes 2-4 are annealing mixtures with ds/ss ratios of 4:1, 2:1, and 1:1, respectively. Lane 5 is the control of ss molecule of pSKdtxR indicated by arrow, the higher molecular weight DNA in this lane is the genomic DNA of the helper phage. Lane 6 is the control of double-stranded plasmid DNA of pSKdtxR. Note that the control double-stranded plasmid DNA is in the supercoiled form, whereas the double-stranded fragment of plasmid pSKdtxR is in the linear form. Lane 7 is the molecular weight marker. **ss**: single-stranded, circular form DNA of phagemid pSKdtxR; **ds**: double-stranded, linear fragment of phagemid pSKdtxR; **ds/ss**: gapped duplex molecule.

ii. Sodium bisulfite mutagenesis Fresh stocks of 50 mM hydroquinone and 4 M sodium bisulfite (pH 6.0) (1.56 g $NaHSO_3$, 0.64 g Na_2SO_3, 4.3 ml H_2O) were usedin mutagenesis. Reaction conditions were determined empirically (Pine and Huang, 1987) with the intent of introducing approximately one C-to-T transition per *dtxR* allele. In each case, 750 ng of gapped duplex DNA in total volume of 450 μl was treated for 10 min in the dark at 37°C with 3 M sodium bisulfite and 2 mM hydroquinone (final concentrations). Reaction mixtures were transferred to dialysis tubing and were dialyzed against sodium phosphate to terminate the reaction. Dialysis was performed three times at 4°C versus one liter volumes of 5 mM potassium phosphate (pH 6.8) with 0.5 mM hydroquinone, for 2 hrs each. Further dialysis was performed against one liter of 200 mM Tris (pH 9.20, 50 mM NaCl, 2 mM EDTA) for 16-20 hrs at room temperature, followed by dialysis against one liter of 10 mM Tris, 1 mM EDTA (pH 8.0) for 4 hrs at 4°C.

iii. Propagation of mutagenized DNA Each sample of mutagenized DNA was precipitated by 95% ethanol and resuspened in 20 μl of deionized H_2O. Gaps on the mutagenized, gapped duplex DNA were filled by using the Klenow fragment of DNA polymerase and T4 ligase, and the completely double-stranded pSKdtxR plasmids were transformed into *E. coli* DH5α(pCMZ100).

5. Oligonucleotide-Directed Site-Specific Mutagenesis

Oligonucleotide-directed site-specific mutagenesis was performed on the antisense stand of the *dtxR* gene as described in the Bio-Rad Muta-Gene manual and by Connell and Holmes (1992) (Fig. 5). The sequences of the sense oligonucleotide primers, which contain mismatched unique or degenerate nucleotides and were used for mutagenesis, were as follows: H98, 5′CAATAAAGTT(A/C)(A/G)CGATGAAGCCT-3′; C102H, 5′-CGATGAAGCCCACCGCTGGG-3′; C102R, 5′-CGATGAAGCCCGCCGCTGGG-3′; C102S, 5′-CGATGAAGCCAGCCGCTGGG-3′; H106, 5′-CCGCTGGGAA(A/C)(A/G)CGTTATGAGT-3′ (underlined base pairs indicate mutations in these primers). Briefly, the phagemid pSKdtxR(-) was transformed in *E. coli* strain CJ236 which contains the *dut* and *ung* double mutations. The single-stranded, uracil-containing DNA (antisense strand) derived from *E. coli* CJ236(pSKdtxR) was isolated with superinfection of helper phage R408 and used as the template for mutagenesis. The oligonucleotide primers were annealed to the templates by heating to 72°C for 5 min and cooling to room temperature for 45 min. The complete, hetero-stranded pSKdtxR plasmids were synthesized in vitro by using the Klenow fragment of DNA polymerase and T4 ligase, which could be detected by electrophoresis on 1.0% agarose gel (Fig. 18, p 91). On the hetero-stranded plasmid, the uracil-containing strand was the wild type, antisense strand DNA of pSKdtxR, and the newly synthesized one was the mutagenized, sense strand DNA. The products of the reactions were then transformed into *E. coli* DH5α(pCMZ100) which contained functional uracil N-glycosylase. In this host strain, the uracil-containing, parental strand was selectively destroyed, and the mutagenized

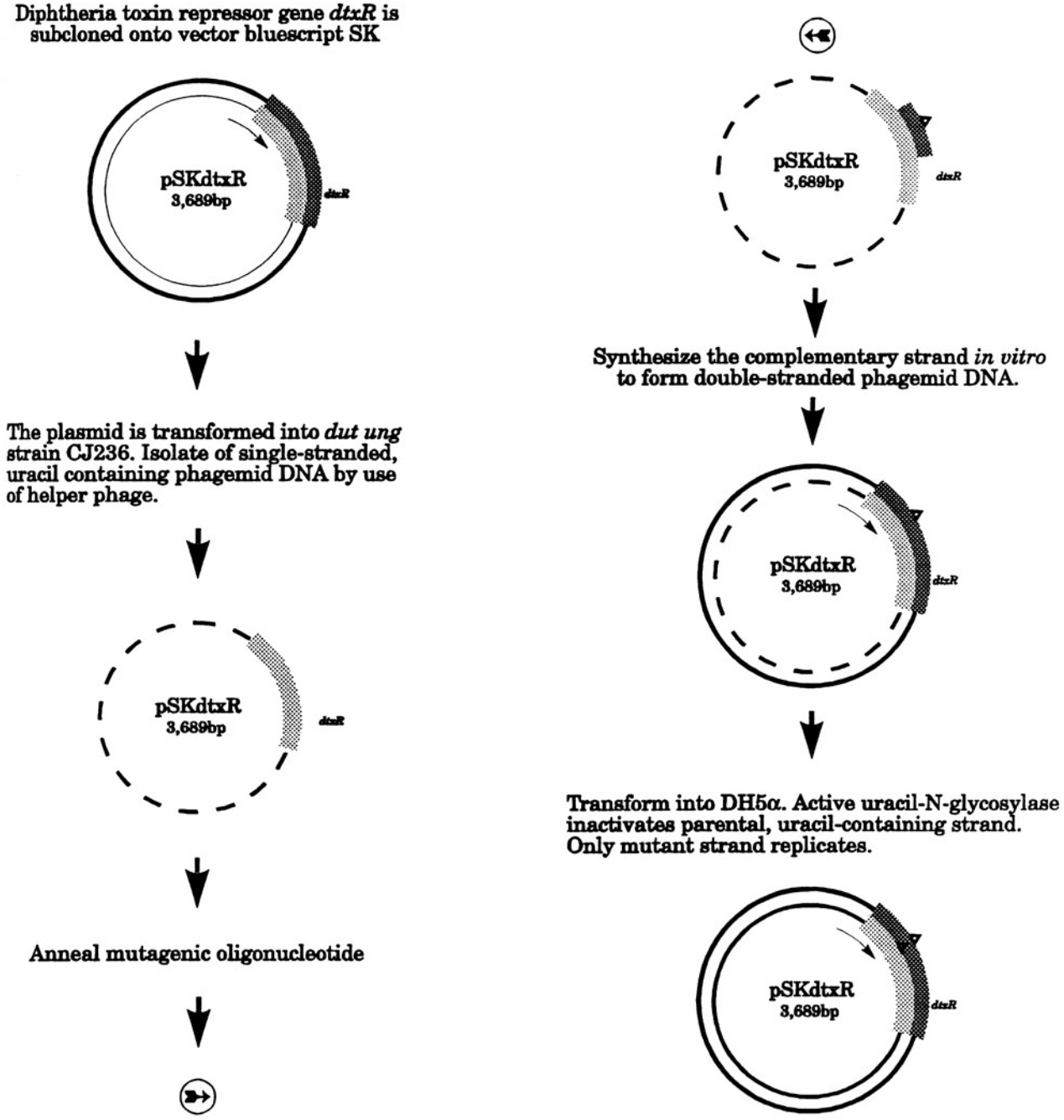


Figure 5. Construction of *dtxR* mutations by oligonucletide-directed site-specific mutagenesis. The wild type *dtxR* gene was subcloned on phagemid pSKdtxR. The uracil containing single-stranded DNA of pSKdtxR was used as the template in oligonucleotide-directed mutagenesis.

strand was amplified by replication of the recombinant phagemid as a plasmid.

6. Strategy for Identification and Isolation of *dtxR* Mutants

i. Identification and isolation of mutants with diminished DtxR repressor activity A reporter plasmid pCMZ100 (Schmitt and Holmes, 1991a) carrying a *tox-lacZ* fusion gene was used to screen the pool of mutagenized *dtxR* alleles. Mutagenized plasmids pSKdtxR, generated from random mutagenesis or site-specific mutagenesis, were transformed into *E. coli* DH5α(pCMZ100). The transformants were grown on LB agar medium containing X-gal and the antibiotics ampicillin and chloramphenicol to maintain positive selection for both plasmids. The presence of the two plasmids, pSKdtxR and pCMZ100, in these transformants was confirmed by electrophoresis on 0.8% agarose gel. A mixture of blue and white colonies appeared on this medium after 24 hrs growth at 37°C. Blue colonies were randomly selected which were defective in DtxR repressor activity, with increasing color indicating progressively greater impairment of repressor function. Colonial phenotypes were designated as follows: -, white; +/-, trace blue; +, light blue; ++, medium blue; +++, dark blue. After two single-colony purifications, the nucleotide sequences of inactivated *dtxR* alleles were determined, and *dtxR* alleles in several white colonies were also sequenced to identify phenotypically $DtxR^+$ strains with silent mutations in the *dtxR* gene (Wang *et al*, 1994). Stock cultures of the isolated strains were stored at -70°C in LB medium containing 20% glycerol.

ii. Identification of mutants with dominant negative phenotypes

Dominant negative *dtxR* mutations were recognized by transforming each of the isolated, mutant pSKdtxR plasmids into host strain *E. coli* DH5α(pCMZ100+pDSK29), and screening for blue colonies on LB agar medium (high-iron conditions) containing X-gal and the antibiotics ampicillin, chloramphenicol, and kanamycin to maintain positive selection for all three plasmids. The presence of all three plasmids in these transformants was confirmed by electrophoresis on 0.8% agarose gel. Blue colonies were scored as described above for intensity of the blue color, and + or ++ indicated that the mutant *dtxR* allele blocked expression of the wild type repressor activity encoded by the $dtxR^+$ allele on plasmid pDSK29.

7. DNA Sequencing and Sequence Analysis

"Double-stranded DNA for sequencing was isolated from the appropriate *E. coli* DH5α clones, and for each clone the sequence of the segment of the *dtxR* gene that had been subjected to mutagenesis was determined by the dideoxy chain termination method of Sanger (Sanger *et al.*, 1977). Dideoxy chain termination reactions were done with T7 polymerase (Sequenase 2.0; United States Biochemical, Cleveland, Ohio) using the following oligonucleotide primers: MCS-1, 5′-ACAAAAGCTGGAGCTCCAC-3′; MW-1, 5′[326]-CTCATAACGTGTTCCCAGC-3′[308]; MCS-2, 5′[158]-TTGTCGTTGTCGCCTCAGA-3′[176]; MW-2, 5′[555]-AGCATCGAGGAGCTGTGTA-3′[537]; MCS-3, 5′[410]-AACTCGGCGTAGGCAATTC-3′[428]; MW-3, 5′-

ATACGACTCACTATAGGGC-3′. The numbers in brackets for MW-1, MCS-2, MW-2, and MCS-3 refer to nucleotide positions within the published DNA sequence of the *dtxR* gene (Boyd *et al.*, 1990). Primers MCS-1 and MW-3 were located within the multicloning site (MCS) of vector pBluescript SK. Reaction products were resolved on 6% polyacrylamide-urea gels (Maniatis *et al.*, 1989)" (Wang *et al*, 1994).

The deduced amino acid sequence of DtxR was analyzed by using the PROTEIN ANALYSIS program (GCG Sequence Analysis Software Package, University of Wisconsin Biotechnology Center, Madison, Wisconsin).

8. Assay of β-Galactosidase Activity

"Because the *tox-lacZ* translational fusion gene in pCMZ100 is negatively regulated by the DtxR repressor, intracellular levels of β-galactosidase were inversely proportional to DtxR repressor activity. The β-galactosidase activity of *E. coli* strains carrying the various plasmids was determined as described previously (Miller, 1972). Briefly, bacterial cultures supplemented with appropriate antibiotics were grown overnight in LB medium with EDDA (low-iron culture) or without EDDA (high-iron culture). 2 ml of the overnight culture was lysed by adding chloroform (100 μl/ml of sample) and 0.1% SDS (50 μl/ml of sample), and 0.9 ml of lysate was transferred to 0.1 ml of Z-buffer at 25°C. The reaction was initiated by adding 200 μl of *o*-nitrophenyl-β-D-galactoside (ONPG) (4 mg/ml). After incubation at 25°C for 10-100 min, the reaction was stopped by adding 0.5 ml of 1 M sodium carbonate. Absorbance was measured at

420 nm and 550 nm using a model DU-64 spectrophotometer (Beckman, Columbia, Md.), and β-galactosidase units were calculated according to Miller (Miller, 1972)" (Wang *et al*, 1994).

9. Western Blot Analysis of DtxR Proteins in Bacterial Extracts

E. coli strains with plasmid pSKdtxR harboring a wild type or mutant *dtxR* allele were grown overnight with aeration at 37°C in LB medium containing ampicillin. Bacteria from 5 ml of overnight culture were harvested by centrifugation at 5,000 ×*g* for 10 min, and subsequent procedures were performed at 4°C. Bacteria were resuspended in 1.5 ml of sonication buffer (10 mM sodium phosphate buffer at pH 7.0, 50 mM NaCl, and 0.02% NaN_3) and disrupted by sonication. Insoluble debris was removed by centrifugation at 25,000 ×*g* for 20 min. 10 µl samples of the crude cell extracts were subjected to 10% SDS/PAGE. The proteins were transferred to nitrocellulose membranes, and the membranes were incubated either with polyclonal rabbit antiserum against a DtxR-MalE fusion protein (Schmitt *et al.*, 1992) or with mouse monoclonal antibody 8G5 against DtxR prepared in our laboratory by E.M. Twiddy (unpublished data). The immobilized rabbit antibodies or mouse antibodies were then treated with an enzyme-labelled second antibody of appropriate specificity, and the blots were developed using a chromogenic substrate as described previously (Holmes and Twiddy, 1983).

10. Purification of DtxR by Ni^{2+}-NTA-Agarose Chromatography

High expression of the *dtxR* gene was accomplished by cloning the modified *dtxR* allele downstream from the strong T7 gene 10 promoter in the vector pBluescript KS (Stratagene, La Jolla, Calif.). The modified *dtxR* allele contains an optimum 9-bp distance between the ribosomal binding site and translational initiation codon of the *dtxR* gene (Schmitt and Holmes, 1993). The *dtxR* gene was transcribed by the heat-inducible T7 RNA polymerase present on plasmid pGP1-2. *E. coli* DH5α containing the plasmid pGP1-2 and pSKdtxR harboring a wild type or mutant *dtxR* allele was grown at 30°C in 50 ml of LB medium with the antibiotics ampicillin and kanamycin to maintain positive selection for both plasmids. When the cultures reached an A_{600} of 1.5, the growth temperature was shifted to 42°C for 30 min. Rifampicin, which specifically inhibits the host RNA polymerase but not the T7 polymerase, was then added to a final concentration of 200 μg/ml, and the cultures were incubated at 37°C for another two hours. Bacteria were harvested by centrifugation at 5,000 ×*g* for 10 min, washed, resuspended in 2.5 ml of sonication buffer (10 mM sodium phosphate buffer at pH 7.0, 50 mM NaCl, and 0.02% NaN_3), and subsequent procedures were performed at 4°C. The cells were disrupted by sonication, and insoluble debris was removed by centrifugation at 25,000 ×*g* for 20 min.

One ml of pre-swollen Ni^{2+}-NTA-agarose resin was packed in the small column supplied with the QIAexpress kit (Qiagen, Chatsworth, Calif.). The column was prepared and equilibrated with sonication buffer as described by manufacturer in the Manual of QIAexpress: the high level expression and protein purification system. A 2 ml sample of each crude cell extract was applied to a separate Ni^{2+}-NTA-column, and columns were

washed with 20 ml samples of sonication buffer. DtxR protein together with a small amount of other proteins were eluted from the columns by step gradients of sonication buffer (1 ml per step) containing increasing concentrations of histidine (1, 2, 3, 5, 7.5, 10, 15, 20, 25, and 50 mM) (Schmitt and Holmes, 1993). Fractions eluted from the column were analyzed by 10% SDS-PAGE, and peak concentrations of wild type and mutant DtxR proteins were obtained in fractions that contained histidine at concentrations between 5 and 20 mM. Fractions were dialyzed against sonication buffer containing Chelex-100 to remove contaminating divalent cations.

11. DNA-Binding Assays of DtxR Proteins

The wild type and variant DtxR proteins purified by Ni^{2+}-NTA-agarose column chromatography were used in these DNA-binding assays. A 330-bp *Eco*RI/*Sal*I fragment carrying the wild type *tox* promoter/operator region was excised from plasmid pPOB (Schmitt *et al.*, 1992), and the fragment was end-labelled with [^{32}P]dCTP at the *Sal*I digestion end by end filling with the Klenow fragment of DNA polymerase. The [^{32}P] labelled DNA fragment was purified by using a Quick Spin column G-50 (Boehringer Mannheim Biochemica, Indianapolis, IN).

i. Gel mobility shift assays End labelled DNA fragments containing the *tox* promoter/operator region at a concentration of ≈0.1 nM were incubated with approximately 0.06 μM wild type or variant DtxR protein in 10-μl reaction volumes in

buffer containing 20 mM Na_2HPO_4 (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, bovine serum albumin (100 μg/ml), sonicated salmon sperm DNA (10 μg/ml), and 10% (vol/vol) glycerol. Ferrous sulfate prepared freshly or salts of other divalent cations were added as indicated. Reaction mixtures were incubated at 25°C for 10 min and loaded (without tracking dye) onto a 5 or 7.5% PAGE gel that contained 20 mM Na_2HPO_4 (pH 7.0) and 1 mM 2-mercaptoethanol. Electrophoresis was performed in 20 mM Na_2HPO_4 (pH 7.0) and 1 mM 2-mercaptoethanol at 75 V for 3 hours using the modular minielectrophoresis system from Bio-Rad. After electrophoresis, the gel was dried and analyzed by autoradiography (Schmitt *et al.*, 1992).

ii. DNase I protection assays End labeled DNA fragments carrying the *tox* promoter/operator region at a concentration of ≈0.5 nM were incubated with approximately 0.06 μM wild type or variant DtxR proteins in 50-μl reaction volumes, in buffer which was identical to that used for the gel mobility shift assays, at 25°C for 15 min. After incubation, the samples were treated with 1 μl of DNase I (Bethesda Research Laboratories) at 10 μg/ml for 1 min at 25°C. Reactions were terminated by extracting with phenol (saturated with Tris at pH 8.0), and then DNA was precipitated with 95% ethanol and dried. Samples were resuspended in 10 μl of 60% formamide buffer containing tracking dye, and electrophoresis was performed through a 6% denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film. To determine precisely the DNA sequences that were protected from DNase I digestion by DtxR protein, Maxam and Gilbert G+A reactions (Maxam and Gilbert, 1980) were also

performed on the labeled fragment (Schmitt *et al.*, 1992).

12. Metal-Binding Assays of DtxR Proteins

The wild type and variant DtxR proteins purified by Ni^{2+}-NTA-agarose column chromatography were used in these metal-binding assays. I used $^{63}Ni^{2+}$, a weak β emitter, as a convenient and suitable isotope for the development of a quantitative assay for the metal-binding activity.

i. $^{63}Ni^{2+}$-binding assays and Scatchard transformation analysis Various amounts of $^{63}Ni^{2+}$ were incubated in 250 µl reaction mixtures with wild type or mutant DtxR protein at concentrations indicated in the text in buffer (10 mM sodium phosphate, pH 7.0, 50 mM NaCl, 1 mM 2-mercaptoethanol) for 15 min at 25°C. Aliquots (200 µl) were filtered through Immobilon™ transfer membranes (Millipore, Bedford, MA) in a Millipore filtration apparatus. Total $^{63}Ni^{2+}$ (T) was determined by counting samples of the reaction mixtures in a Beckman LS-7500 scintillation counter. The amounts of $^{63}Ni^{2+}$ bound to the protein (B) were determined by counting the filter disks, and values for (B) were corrected by subtracting the amounts of $^{63}Ni^{2+}$ bound nonspecifically to disks in samples that contained no DtxR. Statistical significance of observed differences in binding of $^{63}Ni^{2+}$ by wild type DtxR and mutant DtxR proteins was evaluated by *t* tests. Scatchard transformation analysis of the binding data was performed by using software program, LIGAND, Data analysis and curve-fitting for ligand binding experiments (NIH,

Bethesda, MD). The equilibrium dissociation constant (K_d) for high-affinity binding and the maximum number of binding sites were calculated from the Scatchard plots.

ii. Competitive $^{63}Ni^{2+}$-binding assays The ability of other divalent cations to compete with $^{63}Ni^{2+}$ for binding to wild type DtxR was tested by adding a 100-fold molar excess of each divalent cation to be tested into a standard $^{63}Ni^{2+}$-binding assay. In these experiments, 5 μg of DtxR was incubated with 0.592 μM $^{63}Ni^{2+}$ and other divalent cations at 59.2 μM in 250 μl reaction mixtures at 25°C for 15 min. Bound and free $^{63}Ni^{2+}$ were then separated by filtering the samples through Immobilon membranes as described above, and $^{63}Ni^{2+}$ in each fraction was measured by liquid scintillation counting. Statistical significance of observed differences in binding of $^{63}Ni^{2+}$ to wild type DtxR in the presence or absence of other divalent cations was evaluated by *t* tests.

RESULTS

I. Isolation and Characterization of DtxR Variants Induced by Random Mutagenesis with Sodium Bisulfite.

1. Construction of plasmid pSKdtxR containing the wild type *dtxR* allele for mutagenesis.

The goals of my dissertation were to isolate mutant *dtxR* alleles with deficient repressor activity from the cloned, wild type *dtxR* gene in *E. coli*, to purify the variant DtxR proteins encoded by these mutant alleles, and to characterize them by in vitro experiments. However, the relatively low efficiency of transcription from the native *dtxR* promoter in *E. coli* was a limiting factor for purifying and characterizing the variant DtxR proteins. To overcome this problem, the *lacZ* promoter was used to control expression of the cloned *dtxR* gene. Therefore, my project started by constructing plasmid pSKdtxR containing the wild type *dtxR* allele, and pSKdtxR then served as the target for mutagenesis.

Two unique restriction sites on plasmid pMS298 containing the wild type *dtxR* gene (kindly provided by Dr. Michael P. Schmitt, Schmitt and Holmes, 1991a) were used in subcloning, the *Nco*I site which was located at about 30 bp upstream of the translational initiation codon but downstream of the -10 consensus sequence of the *dtxR* promoter, and the *Nar*I site which was located at about 50 bp downstream of the *dtxR* gene (Fig. 6). Plasmid pMS298 was first digested with *Nco*I, end filled by the Klenow fragment of DNA polymerase, and then digested by the second endonuclease *Nar*I. A

Figure 6. Nucleotide and deduced amino acid sequence of the *dtxR* gene.

* indicates -35 and -10 consensus sequences of the *dtxR* promoter. ✠ indicates the ribosomal binding site. Underlined bases indicate the digestion sites for restriction endonucleases *Nco*I and *Nar*I. (Boyd and Murphy, 1990)

```
-150      -100      -90       -80       -70       -60
CAACAAGAAAACTATTCCATATTTTTCACGCTACAATTCGTTGTAGATTGATAGGAATTG
                          ******                ******
-50       -40       -30       -20       -10       1
ATCACCAGCACACAACAGTCTCCATGGCACTATAAGGAAAGAGGCTTACAATGAAGGAC
                     NcoI                           MetLysAsp

10        20        30        40        50        60
TTAGTCGATACCACAGAGATGTACTTGCGTACTATCTATGAGCTGGAAGAAGAGGGAGTC
LeuValAspThrThrGluMetTyrLeuArgThrIleTyrGluLeuGluGluGluGlyVal

70        80        90        100       110       120
ACCCCTCTTCGCGCTAGGATCGCTGAGCGTCTGGAACAATCTGGACCTACAGTTAGCCAA
ThrProLeuArgAlaArgIleAlaGluArgLeuGluGlnSerGlyProThrValSerGln

130       140       150       160       170       180
ACCGTTGCCCGTATGGAGCGCGATGGACTTGTCGTTGTCGCCTCAGACCGCAGTCTACAA
ThrValAlaArgMetGluArgAspGlyLeuValValValAlaSerAspArgSerLeuGln

190       200       210       220       230       240
ATGACACCGACAGGCCGCACTTTAGCGACTGCAGTTATGCGTAAACATCGCTTAGCTGAG
MetThrProThrGlyArgThrLeuAlaThrAlaValMetArgLysHisArgLeuAlaGlu

250       260       270       280       290       300
CGCCTTCTTACCGATATCATTGGTCTAGATATCAATAAAGTTCACGATGAAGCCTGCCGC
ArgLeuLeuThrAspIleIleGlyLeuAspIleAsnLysValHisAspGluAlaCysArg

310       320       330       340       350       360
TGGGAACACGTTATGAGTGACGAAGTTGAACGCAGGCTCGTGAAAGTATTGAAAGATGTC
TrpGluHisValMetSerAspGluValGluArgArgLeuValLysValLeuLysAspVal

370       380       390       400       410       420
AGTCGGTCCCCCTTCGGAAACCCAATTCCAGGTCTCGACGAACTCGGCGTAGGCAATTCT
SerArgSerProPheGlyAsnProIleProGlyLeuAspGluLeuGlyValGlyAsnSer

430       440       450       460       470       480
GACGCGGCAGCCCCCGGAACTCGCGTTATTGACGCTGCCACCAGCATGCCCCGCAAAGTA
AspAlaAlaAlaProGlyThrArgValIleAspAlaAlaThrSerMetProArgLysVal

490       500       510       520       530       540
CGCATTGTTCAGATTAACGAAATCTTTCAAGTTGAAACGGATCAGTTTACACAGCTCCTC
ArgIleValGlnIleAsnGluIlePheGlnValGluThrAspGlnPheThrGlnLeuLeu

550       560       570       580       590       600
GATGCTGACATCCGTGTTGGATCAGAAGTCGAAATTGTAGATAGAGACGGCCACATCACG
AspAlaAspIleArgValGlySerGluValGluIleValAspArgAspGlyHisIleThr

610       620       630       640       650       660
TTGAGCCACAATGGAAAAGATGTCGAACTCCTCGATGATCTGGCTCACACTATTCGTATC
LeuSerHisAsnGlyLysAspValGluLeuLeuAspAspLeuAlaHisThrIleArgIle

670       680       690       700       710       720
GAAGAACTCTAAATACTAAAGGCGGCGAAATTAGATGAAACTCCTCGTTACCGGTGGCGCC
GluGluLeu!!!                                          NarI
```

760-bp DNA fragment containing the wild type *dtxR* allele without its native promoter was generated and isolated after these treatments. The fragment was then ligated to *Sma*I/*Cla*I cut pBluescript SK(+) or SK(-) vectors, and the newly constructed plasmids were designated as pSKdtxR(+) or pSKdtxR(-), respectively (Fig. 7). The map of plasmid pSKdtxR is shown in Figure 8. The construction of plasmid pSKdtxR was analyzed and confirmed by restriction endonuclease digestion and electrophoresis on a 1.0% agarose gel (Fig. 9). Colonies of *E. coli* DH5α(pSKdtxR) on LB agar medium containing X-gal were white, in contrast to the blue colonies formed by *E. coli* DH5α(pBluescript SK). The nucleotide sequence of the wild type *dtxR* allele in plasmid pSKdtxR was verified by DNA sequencing. The repressor activity of the $dtxR^+$ allele in the plasmid pSKdtxR was tested in host strain *E. coli* DH5α containing the reporter plasmid pCMZ100 (Fig. 10). *E. coli* DH5α(pSKdtxR+pCMZ100) gave white colonies on LB agar medium containing X-gal (high-iron conditions) and blue colonies on LB agar medium containing X-gal and EDDA (low-iron conditions) (Fig. 11). These observations demonstrated that plasmid pSKdtxR harbored a functional $dtxR^+$ allele.

2. Bisulfite mutagenesis

Plasmids pSKdtxR(+) and pSKdtxR(-), derived from the pBluescript SK(+) and SK(-) phagemid vectors, respectively, were transformed into *E. coli* strain XL1-Blue and used to generate single-stranded DNA templates for each strand of the *dtxR* gene (Fig. 12). These single-stranded DNA templates were used to construct gapped duplex DNA molecules. To restrict the target for mutagenesis and facilitate sequencing of the mutant

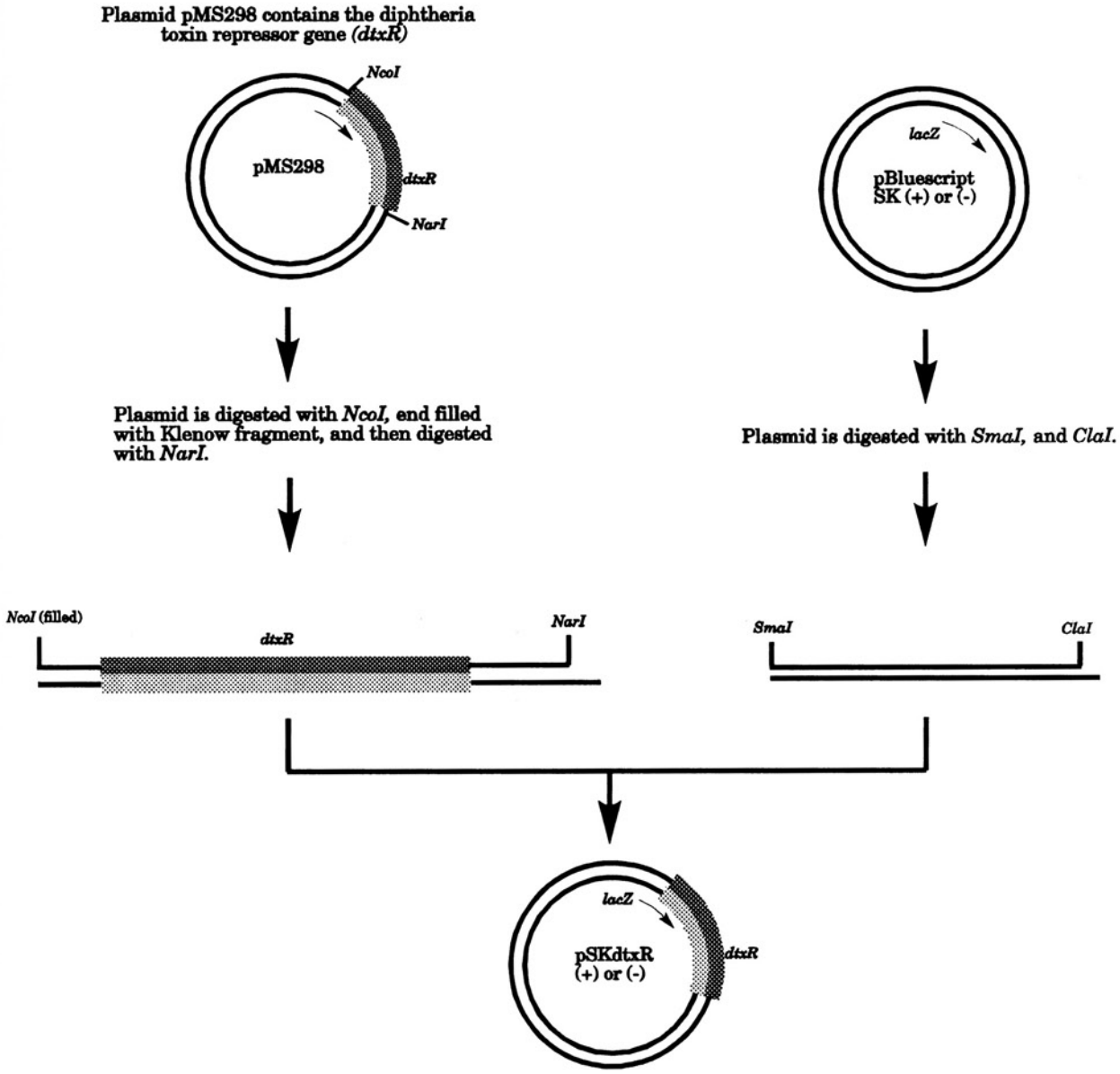


Figure 7. Schematic diagram for the construction of plasmid pSKdtxR (+) & pSKdtxR (-).

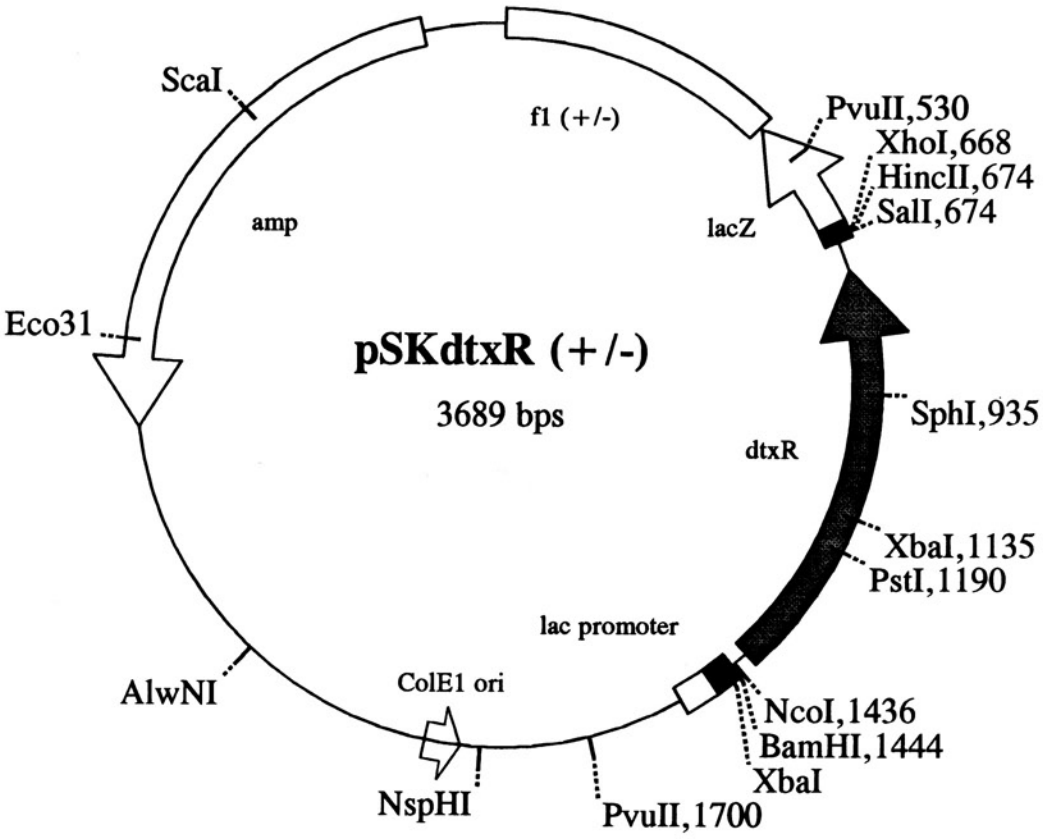


Figure 8. Physical map of plasmid pSKdtxR. A 760-bp DNA fragment containing the wild type *dtxR* gene was inserted into the multicloning site of pBluescript SK (+) and pBluescript SK (-) vectors. Expression of the *dtxR* gene is under the control of the *lac* promoter on the vectors, and transcription of *dtxR* procedes counterclockwise from the *lac* promoter. The native *dtxR* promoter is deleted.

Figure 9. Restriction analysis of plasmids pSKdtxR(+) and pSKdtxR(-). Lanes 1-3 are plasmids pSKdtxR (+). Lanes 5-7 are pSKdtxR(-); and lanes 4 and 8 are molecular weight markers. Lanes 1 and 5 are plasmids without digestion; lanes 2 and 6 are *Bam*HI and *SalI* double digests; and lanes 3 and 7 are *Xba*I single digests. Samples were analyzed by electrophoresis on a 1.2% agarose gel.

Figure 10. Regulation of the *tox-lacZ* fusion gene by DtxR in *E. coli*. The wild type *dtxR* gene is located on plasmid pSKdtxR, and the *tox-lacZ* reporter gene is located on plasmid pCMZ100. **a)** Under high-iron conditions, the DtxR-iron complex binds to the *tox* operator region and prevents transcription from the *tox-lacZ* gene. The stoichiometry of iron binding by DtxR has not been established. **b)** Under low-iron conditions, the aporepressor DtxR can not bind to the *tox* operator and fails to repress transcription from the *tox* promoter. β-galatosidase is expressed under these conditions.

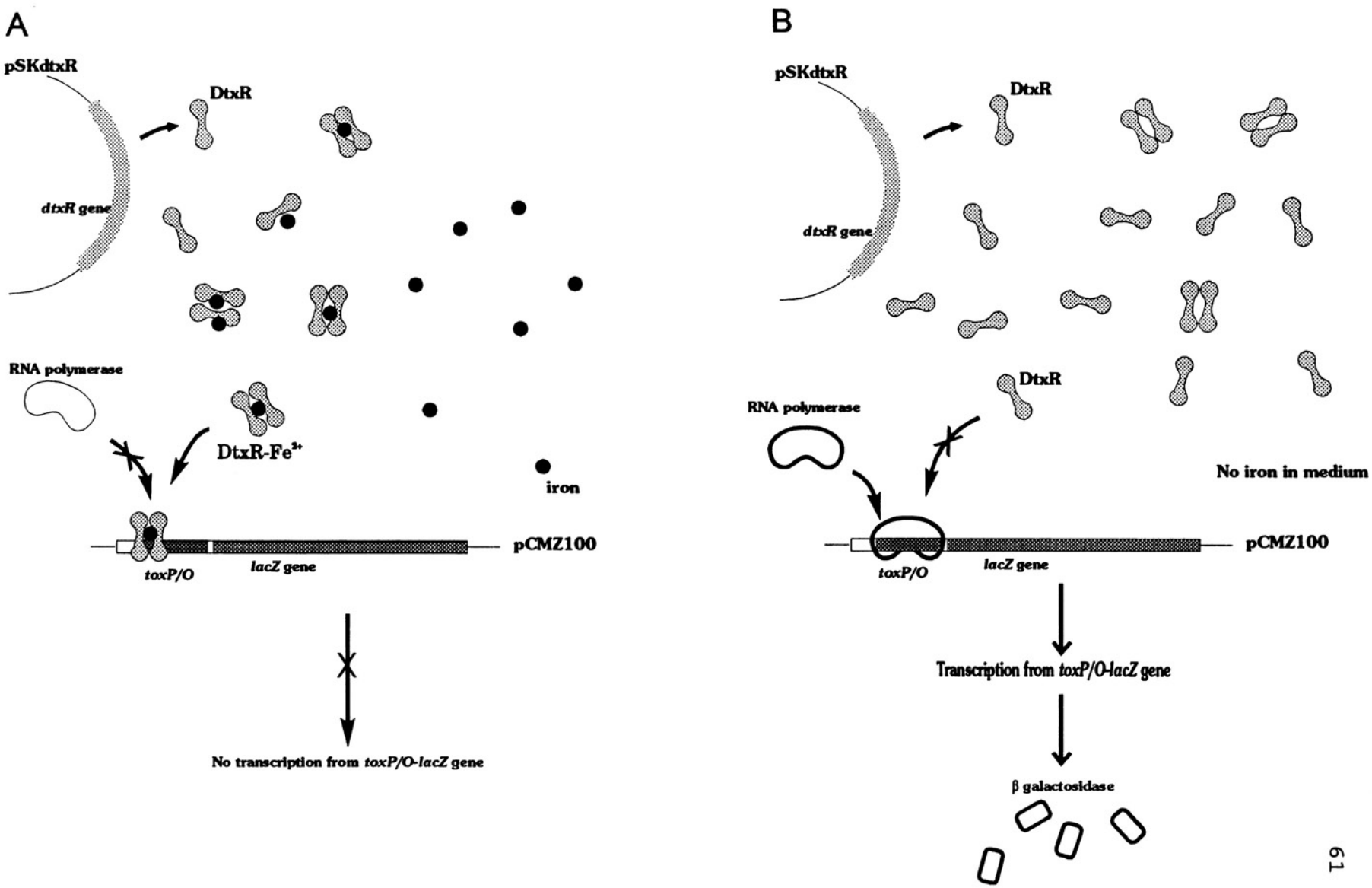

A
pSKdtxR
DtxR
dtxR gene
RNA polymerase
DtxR-Fe3+
iron
pCMZ100
toxP/O
lacZ gene
No transcription from toxP/O-lacZ gene
B
pSKdtxR
DtxR
dtxR gene
DtxR
RNA polymerase
No iron in medium
pCMZ100
toxP/O
lacZ gene
Transcription from toxP/O-lacZ gene
β galactosidase

Figure 11. The colonial phenotypes of *E. coli* DH5α(pSKdtxR+pCMZ100) strains harboring wild type and mutant *dtxR* alleles on LB agar medium containing X-gal.

E. coli DH5α containing the reporter plasmid pCMZ100, which carries a *tox-lacZ* gene fusion under transcriptional control of the *tox* promoter-operator region, formed blue (Lac^+) colonies on both LB agar medium (high-iron conditions) containing X-gal and LB agar medium containing X-gal and EDDA (low-iron conditions) (data not shown). When the wild type *dtxR* gene, present on the multicopy plasmid pSKdtxR, was transformed into DH5α(pCMZ100), white colonies (Lac^-) were formed on LB medium, which indicated that transcription of the reporter gene from the *tox* promoter was repressed under high-iron conditions, and blue colonies (Lac^+) were formed on LB medium with EDDA, which indicated derepression of the *tox* promoter by DtxR under low-iron condition (WT = wild type). If a repressor-deficient mutant *dtxR* allele such as H106Y was present on plasmid pSKdtxR, colonies were blue on both LB and LB(EDDA) agar media.

Figure 12. Isolation of single-stranded DNA of phagemid pSKdtxR from *E. coli* strain XL1-Blue. Single-stranded DNA was examined on a 1.0% agarose gel. Lane 1 is the molecular weight marker. Lane 2 is double-stranded plasmid pSkdtxR. Lanes 3 and 4 are single-stranded DNA of pSKdtxR (+) and pSKdtxR (-), respectively. Lane 5 is the control of single-standed DNA of vector pBluescript SK. Lane 6 is the control single-stranded DNA prep from XL1-Blue (without plasmid) superinfected with helper phage R408. **ss** indicates the single-stranded DNA of phagemid pSkdtxR.

dtxR alleles obtained, three sets of gapped duplex molecules were prepared. The 678 bp *dtxR* gene was divided into three regions, with the single-stranded segments of the gapped duplex molecules corresponding with an N-terminal region (-30 to 280 bp, *Xba*I-*Xba*I), a central region (220 to 470 bp, *Pst*I-*Sph*I), and a C-terminal region (470 to 700 bp, *Sph*I-*Sal*I) (Fig. 13). The gapped duplex molecules were mutagenized with sodium bisulfite and transformed into *E. coli* DH5α(pCMZ100). The transformants carrying the mutagenized *dtxR* genes were then tested for the production of β-galactosidase from the *tox-lacZ* fusion gene to determine the phenotypes associated with their *dtxR* alleles (Fig. 14).

3. Identification and in vivo characterization of *dtxR* mutations

i. Phenotypes of mutagenized *dtxR* alleles

Each of the three regions of the *dtxR* gene on each strand was subjected to bisulfite mutagenesis, and greater than 800 transformants mutagenized within each of the three regions were screened. Among transformants that had been subjected to mutagenesis in the segment that encoded the N-terminal region, the central region, and the C-terminal region of DtxR, blue colonies represented 55%, 51%, and 21% , respectively, of the total colonies (Table 4). Three sets of clones, each consisting of 30 blue colonies and 4 white colonies representing a different mutagenized region of the *dtxR* gene, were selected randomly and purified by single colony isolations, and the nucleotide sequences of the *dtxR* alleles in the 102 selected clones were determined. Strains were excluded from further analysis if they contained mutations in more than one codon or if

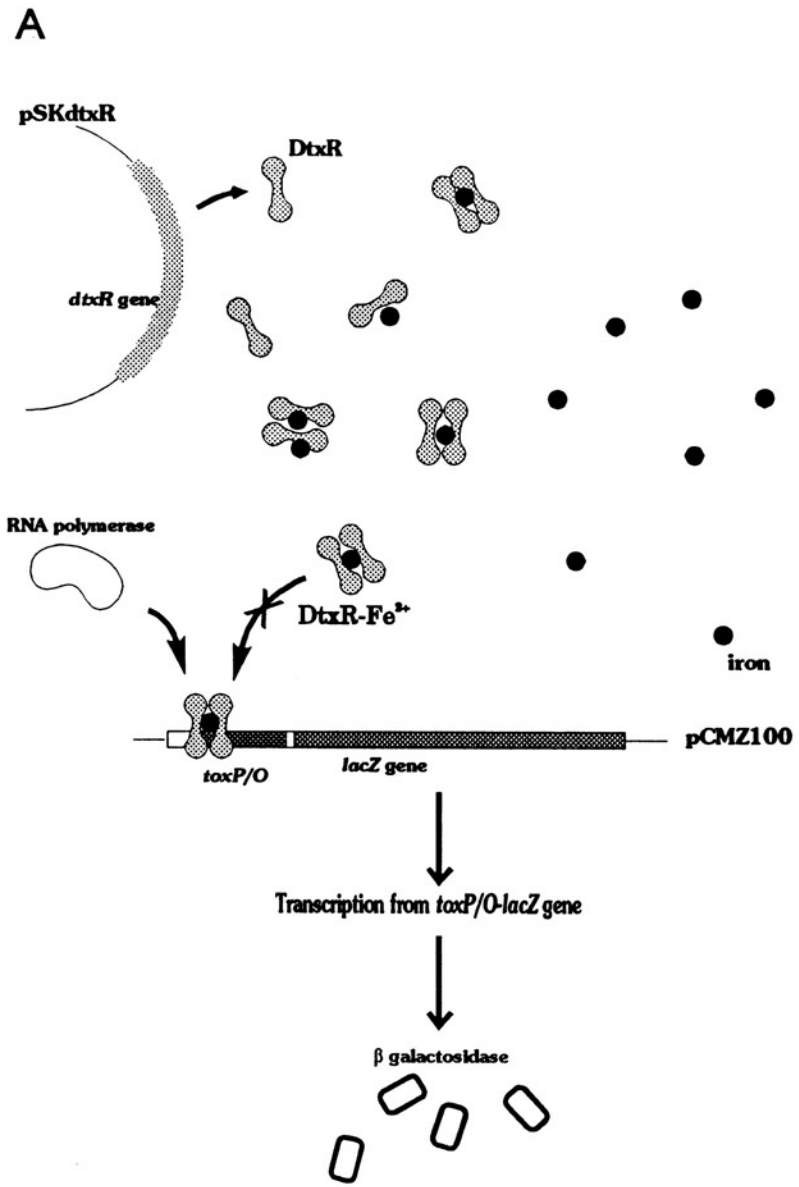
Figure 13. Detection of the gapped duplex molecules by electrophoresis on 1.0% agarose gels.

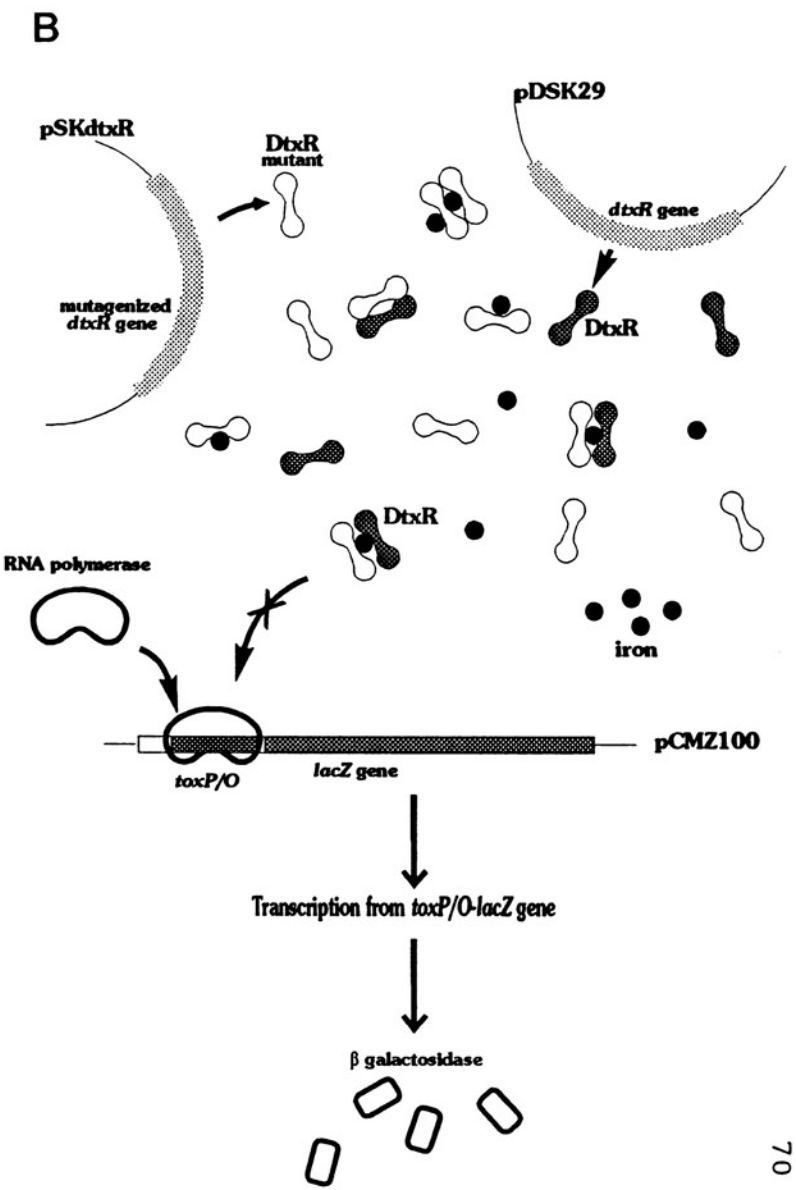
Upper panel: lane 1 is the molecular weight marker; lane 2 is double stranded plasmid pSKdtxR; lanes 3 and 5 are the annealing products of the dsDNA fragment of pSKdtxR (*Xba*I/*Xba*I) and ssDNA pSKdtxR (+) and pSKdtxR (-), respectively; lanes 4 and 6 are the reaction mixtures without annealing that correspond with lanes 3 and 5, respectively; lane 7 is the dsDNA fragment pSKdtxR (*Xba*I/*Xba*I); lane 8 is the single-stranded DNA prep of pSKdtxR.

Lower panel: lane 1 is the molecular weight marker; lane 2 is double stranded plasmid pSKdtxR; lanes 3 and 4 are the annealing products of dsDNA fragment of pSKdtxR (*Pst*I/*Sph*I) and ssDNA of pSKdtxR (+) and pSKdtxR (-), respectively; lanes 5 and 6 are the annealing products of dsDNA fragment of pSKdtxR (*Sph*I/*Sal*I) and ssDNA of pSKdtxR (+) and pSKdtxR (-), respectively; lane 7 is the dsDNA fragment pSKdtxR (*Pst*I/*Sph*I); lane 8 is the single-stranded DNA prep of pSKdtxR.

ds, double-stranded DNA; **ss**, single-stranded DNA; **ds/ss**, gapped duplex DNA.

Figure 14. Diagram of identification of *dtxR* mutations. **A**) Identification of *dtxR* mutations with defective repressor activity in *E. coli* DH5α(pSKdtxR+pCMZ100). Under high-iron conditions, nonfunctional DtxR variants will fail to repress the *tox* promoter, resulting in production of β-galactosidase. **B**) Identification of *dtxR* mutations with dominant negative phenotype in *E. coli* DH5α(pSKdtxR+pCMZ100+pDSK29). Under high-iron conditions, DtxR variants interfere with the repressor function of wild type DtxR, resulting in production of β-galactosidase.


A
pSKdtxR
DtxR
dtxR gene
RNA polymerase
DtxR-Fe3+
iron
pCMZ100
toxP/O
lacZ gene
Transcription from toxP/O-lacZ gene
β galactosidase


B
pSKdtxR
DtxR
mutant
pDSK29
dtxR gene
mutagenized
dtxR gene
DtxR
DtxR
RNA polymerase
iron
pCMZ100
toxP/O
lacZ gene
Transcription from toxP/O-lacZ gene
β galactosidase

a. +, sense strand DNA of the *dtxR* gene; -, antisense strand DNA of the *dtxR* gene.

b. The colonial phenotype indicates the number of blue colonies (nonfunctional, mutant *DtxR* gene) and white colonies (functional wild type or mutant *dtxR* gene) recovered among bacteria transformed with mutagenized stocks of pSKdtxR.

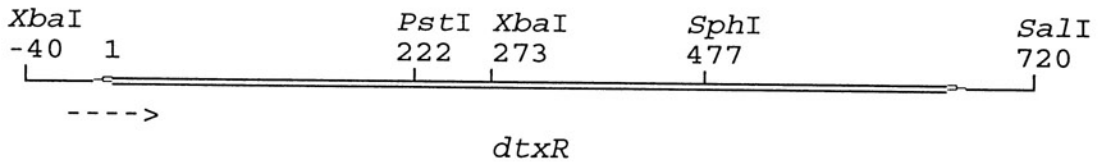


Table 4. Results of Random Mutagenesis by Sodium Bisulfite

Gapped Duplex	Length (bp)	DNA Strand[a]	Cytosine Residues	Colonial Phenotype[b]			Blue/Total (%)
				Blue	White	Total	
*Xba*I-*Xba*I	273	+	71	365	231	596	
		-	70	160	192	352	
		subtotal	141	525	423	948	55.4
*Pst*I-*Sph*I	255	+	67	224	298	522	
		-	65	231	162	393	
		subtotal	132	465	450	915	50.8
*Sph*I-*Sal*I	201	+	48	130	436	566	
		-	43	42	215	257	
		subtotal	91	172	651	823	20.9

they represented independent isolates of *dtxR* alleles already identified. In this manner, twenty different mutant *dtxR* alleles with alterations in single codons were identified, two of which resulted in premature termination. Characterization of these 20 mutant *dtxR* alleles is summarized in Table 5.

DtxR variants with single amino-acid substitutions were designated by the one-letter code for the wild type amino acid, its number in the sequence of the DtxR polypeptide, and the one-letter code for the amino acid at that position in the mutant polypeptide (e.g., T67I for the variant with isoleucine replacing threonine at residue number 67). T67I and T24I were phenotypically indistinguishable from wild type DtxR. They are encoded by *dtxR* alleles with silent mutations identified among the strains that retained the ability to form white colonies on LB agar plates containing X-gal. All variants of DtxR that exhibited decreased repressor activity were from strains that formed blue colonies on LB agar plates with X-gal (Fig. 11, p 63). Rank ordering of the strains based on quantitative assays for β-galactosidase activity correlated well with rank ordering based qualitatively on intensity of the blue color of the colonies. All of the mutants may retain at least trace levels of repressor activity, since the β-galactosidase activity observed in the presence of the negative control plasmid pSKlac$^-$ (51.9 units) was greater than that observed with any of the *dtxR* mutants.

The variant R47H contains the same mutation as the nonfunctional *dtxR* allele isolated from *C. diphtheriae* mutant strain C7hm723(β)$^{tox+}$, which produced diphtheria toxin constitutively (Schmitt and Holmes, 1991b; Kanei *et al.*, 1977). The β-galactosidase assays in *E. coli* also revealed that R47H retained a low level of repressor

a. Functional assays for repressor included colonial phenotype and quantitative measurement of β-galactosidase (β-gal) activity. Increasing colonial color or enzyme activity indicates progressively greater impairment of repressor function. The control value for β-galactosidase activity in *DH5α*(pCMZ100) without a second or third plasmid was 86.0 ±7.1.

b. Strain designations reflect the amino acid substitution or premature chain termination that occurs in the DtxR protein encoded by the *dtxR* allele in the mutant pSKdtxR plasmid.

c. *E. coli DH5α* contains a *supE* allele that permits production of some W104E as well as the truncated protein W104.

Table 5. Characterization of Mutant *dtxR* Alleles Isolated from Random Mutagenesis

STRAIN	CODON CHANGE in *dtxR*	FUNCTIONAL ASSAY[a]	
		PHENOTYPE	β-gal ACTIVITY
Mutants of pSKdtxR[b]			
T67I	ACA → ATA	-	0.3 ±0.3
T24I	ACC → ATC	-	0.3 ±0.2
E19K	GAA → AAA	+/-	0.8 ±0.3
T44I	ACC → ATT	+/-	0.8 ±0.2
T7I	ACC → ATC	+/-	1.3 ±0.4
R47H	CGT → CAT	+	2.0 ±0.1
A72V	GCG → GTG	+	2.6 ±0.1
R13C	CGT → TGT	+	2.7 ±0.5
R84H	CGC → CAC	+ +	4.8 ±0.6
D88N	GAT → AAT	+ +	5.7 ±1.6
R77H	CGT → CAT	+ +	7.1 ±0.8
W104[c]	TGG → TAG	+ + +	9.5 ±0.1
T40I	ACA → ATA	+ + +	11.7 ±1.1
A147V	GCC → GTC	+ + +	11.9 ±0.9
H106Y	CAC → TAC	+ + +	12.0 ±0.3
E100K	GAA → AAA	+ + +	12.6 ±1.9
P39L	CCT → CTT	+ + +	14.1 ±1.2
Q36	CAA → TAA	+ + +	17.1 ±1.8
G52E	GGA → GAA	+ + +	17.7 ±1.9
A46V	GCC → GTC	+ + +	28.8 ±1.9
Control plasmids			
pSKdtxR		-	0.2 ±0.2
pDSK29		-	0.3 ±0.2
pSKlac⁻		+ + +	51.9 ±4.1

activity, consistent with the slightly leaky repressor-defective phenotype of *C. diphtheriae* C7hm723(β^{tox+}).

ii. Dominance of dtxR alleles

Mutant forms of repressor that lack activity and also interfere with activity of the wild type repressor protein have been obtained for several *E. coli* repressors (Kelley and Yanofsky, 1985; Teliveris and Mount, 1992). This dominant-negative phenotype could result either from formation of nonfunctional heterodimers of the mutant and wild type repressor polypeptides or, less probably, from competition between homodimers of the nonfunctional mutant polypeptides and wild type repressor. We therefore tested the nonsense and missense mutants of *dtxR* to determine if any expressed a dominant-negative phenotype. To optimize the sensitivity of these tests, we used plasmid pDSK29, a low-copy number plasmid that contains the wild type *dtxR* allele, to repress the *tox-lacZ* reporter gene of pCMZ100 (Fig. 14, p 70). Since the mutant *dtxR* genes were present on the high-copy number plasmid pSKdtxR, there should be a large excess of mutant monomers over wild-type monomers. Random association of the mutant and wild type subunits should result in relatively few wild type repressor complexes being formed. Reduced repressor activity of the transformants containing the mutant and wild type heterodimers or multimers should be reflected by an increase in expression of the *tox-lacZ* fusion gene. Therefore, blue colonies of these transformants on LB (X-gal) plates should indicate that the strain expressed a mutant DtxR protein that interfered with the normal repressor function.

Each mutant pSKdtxR plasmid was transformed into DH5α(pCMZ100+pDSK29), and the transformants were screened on LB agar containing X-gal. Twelve of the 16 *dtxR* missense mutants with deficient repressor activity expressed the dominant negative phenotype in this assay system (Table 6).

During an independent random mutagenesis experiment aiming to isolate additional DtxR variants with dominant negative phenotypes, the pools of mutagenized plasmid pSKdtxR were transformed directly into DH5α(pCMZ100+pDSK29). One such mutant *dtxR* allele causing a single-amino-acid substitution in DtxR was isolated and sequenced from this experiment. This variant contained a tyrosine substitution for histidine at position 106, the same amino acid replacement in variant H106Y isolated from previous random mutagenesis.

4. Intracellular expression of mutant *dtxR* alleles

The intracellular level of each of the mutant repressor proteins was analyzed by Western blotting analysis (Fig. 15). Most of the defective DtxR proteins were immunoreactive, were produced at levels comparable to wild type DtxR, and had the same mobility in SDS-PAGE as the wild type DtxR. R13C, A46V, and W104Q had slightly slower electrophoretic mobilities. No immunoreactivity was detected for A147V or the truncated polypeptide Q36 or W104 (from *E. coli* strain MC1009), and G52E gave a weak reaction. An immunoreactive variant W104Q was produced from the plasmid encoding W104 variant in *E. coli* DH5α containing *supE* mutation. These results indicated that most of the mutant repressors were present in *E. coli* in amounts comparable to wild type

a. Increasing colonial color indicates progressively greater inhibition of wild-type DtxR.

b. *E. coli DH5α* contains a *supE* allele that permits production of some W104E as well as the truncated protein W104.

c. ND = not determined

Table 6. Dominance of Mutant *dtxR* Alleles.

STRAIN	DOMINANCE OF *dtxR* ALLELE[a]
Mutants of pSKdtxR	
T67I	-
T24I	-
E19K	-
T44I	+/-
T7I	-
R47H	+
A72V	+/-
R13C	-
R84H	+/-
D88N	+/-
R77H	+
W104[b]	+
T40I	+
A147V	-
H106Y	+
E100K	+ +
P39L	+
Q36	-
G52E	+
A46V	-
Control plasmids	
pSkdtxR	-
pDSK29	ND[c]
pSKlac⁻	ND

Figure 15. Western blot analysis of mutant forms of DtxR. Extracts of *E. coli* DH5α containing pSKdtxR plasmids with wild type or mutant *dtxR* alleles were subjected to 10% SDS-PAGE. The plasmid encoding the W104 variant of DtxR was also tested in the suppressor-negative *E. coli* strain MC1009(*supE*). The separated proteins were transferred to nitrocellulose membranes, and the DtxR proteins on the membrane were allowed to react with **A**) polyclonal rabbit antibody against DtxR-MalE fusion protein, or **B**) a monoclonal mouse anti-DtxR antibody. The immobilized antibodies were then allowed to react with an enzyme-labelled second antibody, and the blots were developed using a chromogenic substrate for the enzyme activity (peroxidase).

DtxR. With the possible exception of G52E and A147V, the mutant phenotypes of the DtxR variants with single amino acid substitutions were unlikely to result from decreased synthesis or accelerated degradation in comparison with wild type DtxR.

5. Distribution of amino acid substitutions in DtxR.

Seventeen of the 18 single amino acid substitutions that resulted in decreased repressor activity were located within the amino-terminal half of DtxR (residues 1-110). The distribution of these replacements was not random, instead, they clustered into four groups (Fig. 16).

A functional *dtxR* allele isolated from *C. diphtheriae* 1030(-) strain contained six amino acid substitutions within the C-terminal region (Boyd *et al.*, 1992). This finding suggested that the C-terminal half of DtxR might be less important than the N-terminal half for repressor activity. Our results showing that single amino acid substitutions in the N-terminal half of DtxR were more likely to cause decreased repressor activity (Wang *et al.*, 1994) were consistent with this conclusion.

Computer analysis revealed a predicted helix-turn-helix-turn-helix secondary structure at the amino-terminus (residues 1-52). Nine of the repressor-deficient variants have substitutions within these 52 residues. The predicted helix-turn-helix motif between residues 28 and 52 was found to be similar to DNA-binding motifs in several other well characterized proteins (Fig. 17) (Brennan and Mathews, 1989; Harrison, 1991; Sauer *et al.*, 1982). Six of the amino acid substitutions (Group II) in DtxR are clustered in the segment between residues 39 and 52, including five that caused dominant-negative

Figure 16. Amino acid sequence of the amino-terminal half of wild-type DtxR (1-120 aa.) and positions of amino acid substitutions in mutant forms of DtxR. Predicted secondary structures and functional domains are *bracketed. Triangle* indicates an amino acid substitution that resulted in defective repressor activity; *circle* indicates a substitution that had no effect on repressor activity. The *letter* within a *triangle* or *circle* indicates the amino acid residue substituted for the wild type residue. A *diamond* within a *triangle* indicates a chain-terminating mutant. *Arrows* indicate the mutants with dominant-negative phenotypes. *Solid* and *shaded arrows*, respectively, represent strong and weak interference with the repressor activity of wild-type DtxR.

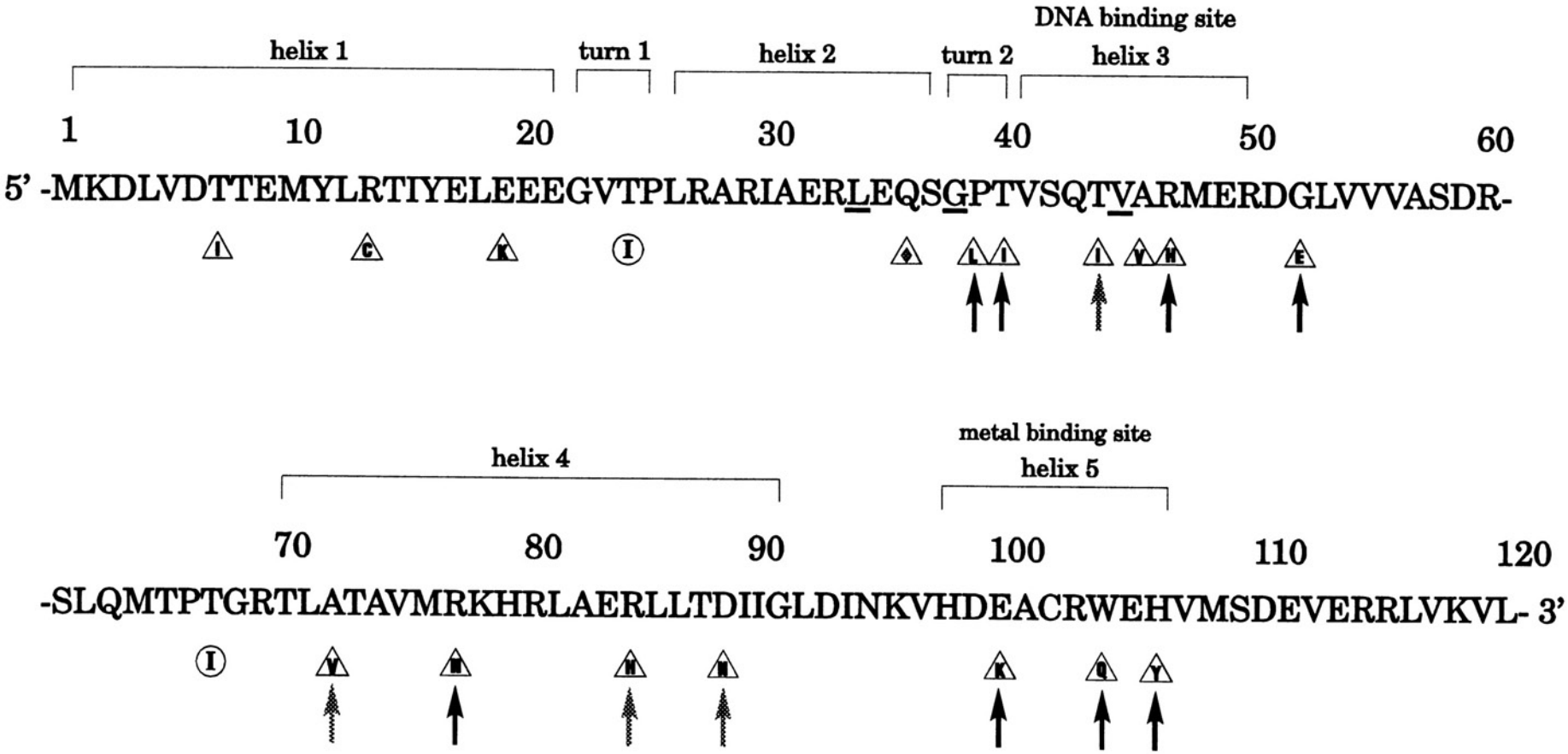
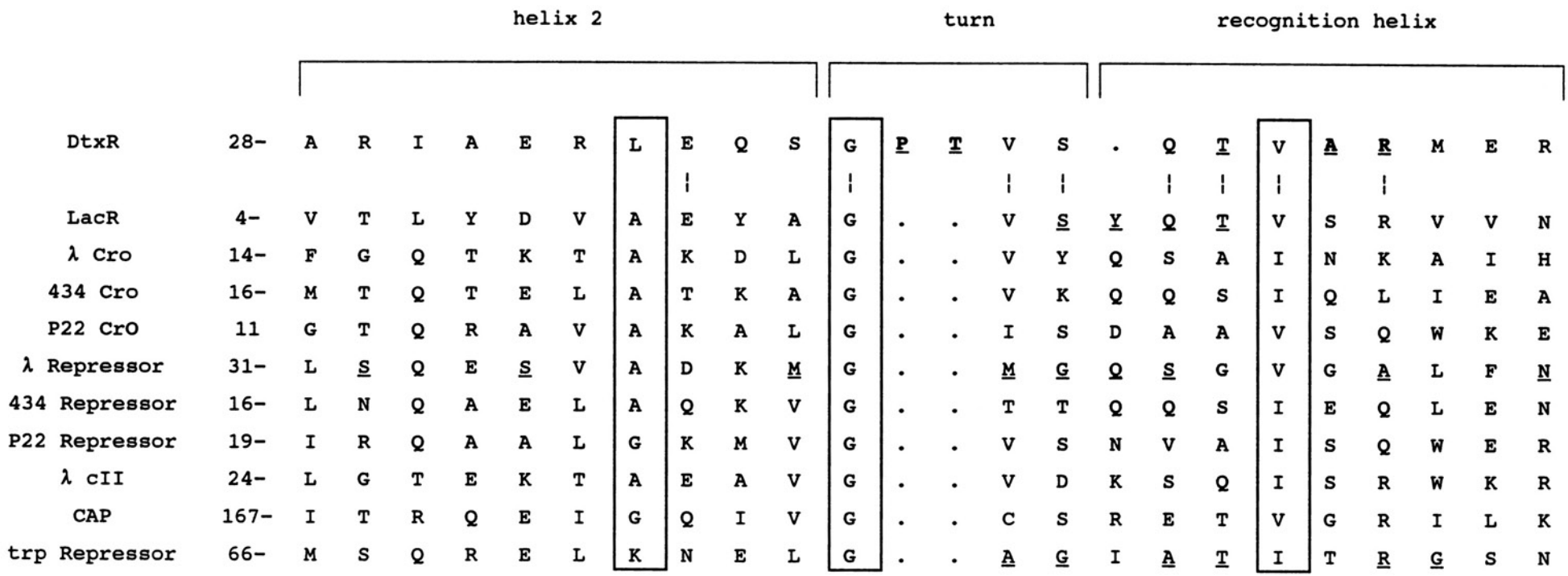

DNA binding site
helix 1
turn 1
helix 2
turn 2
helix 3
1
10
20
30
40
50
60
5' -MKDLVDTTEMYLRTIYELEEEGVTPLRARIAERLEQSGPTVSQTVARMERDGLVVVASDR-
helix 4
metal binding site
helix 5
70
80
90
100
110
120
-SLQMTPTGRTLATAVMRKHRLAERLLTDIIGLDINKVHDEACRWEHVMSDEVERRLVKVL- 3'

Figure 17. Comparison of residues 28 through 50 of DtxR with the helix-turn-helix motifs of several other DNA-binding proteins. The sequences shown are from references Boyd *et al.*, 1990, Brennan and Matthews, 1989, and Harrison, 1991. The most highly conserved residues are *boxed*. *Vertical bars* between the DtxR and LacR sequences indicate identical residues. The predicted helix 3 of DtxR (Fig. 16) appears to correspond with the DNA recognition helixes of the other repressor proteins. *Underlined residues* in DtxR and the other repressors indicate the positions at which amino acid substitutions abolished or diminished repressor activity.


helix 2
turn
recognition helix
DtxR 28- A R I A E R L E Q S G P T V S . Q T V A R M E R
LacR 4- V T L Y D V A E Y A G . . V S Y Q T V S R V V N
λ Cro 14- F G Q T K T A K D L G . . V Y Q S A I N K A I H
434 Cro 16- M T Q T E L A T K A G . . V K Q Q S I Q L I E A
P22 CrO 11 G T Q R A V A K A L G . . I S D A A V S Q W K E
λ Repressor 31- L S Q E S V A D K M G . . M G Q S G V G A L F N
434 Repressor 16- L N Q A E L A Q K V G . . T T Q Q S I E Q L E N
P22 Repressor 19- I R Q A A L G K M V G . . V S N V A I S Q W E R
λ cII 24- L G T E K T A E A V G . . V D K S Q I S R W K R
CAP 167- I T R Q E I G Q I V G . . C S R E T V G R I L K
trp Repressor 66- M S Q R E L K N E L G . . A G I A T I T R G S N

phenotypes and four that resulted in quantitatively severe deficits in repressor function (+++ colonial phenotypes and β-galactosidase activity >10). In contrast, the amino acid substitutions (Group I) located within the first 25 residues at the amino-terminus of DtxR caused only moderate to negligible decreases in repressor activity. The region between residues 39 and 52 is an excellent candidate for the domain that recognizes and interacts with DtxR-regulated operators.

The His98-XXX-Cys102-XXX-His106 sequence has a predicted helical secondary structure and similarity with metal-binding motifs found in several other proteins (Arnold and Haymore, 1991). Three of the DtxR variants (Group IV) (E100K, W104Q, and H106Y), all with severe deficits in repressor function and strong dominant negative phenotypes, have amino acid substitutions within this motif. The region between residues 98 and 106 is, therefore, an excellent candidate for the metal-binding domain involved in activation of DtxR by Fe^{2+} or other divalent metal ions.

Four DtxR variants (Group III) with moderate deficiencies in repressor activity were located in a sequence between the presumed DNA-binding and metal-binding site that contains a predicted long helix (residue 70-90). The role of this region in the function of DtxR is currently unknown.

Amino acid substitutions associated with the dominant negative phenotype were clustered in three of four regions of DtxR described above, between residues 39-52, 70-90, and 98-106.

II. Construction and Analysis of Additional DtxR Variants Containing Amino Acid Substitutions within the Putative Metal-Binding Domain

1. Isolation of site-directed mutations

Histidine and cysteine are two of the most common metal-coordinating residues found in metal-binding proteins (Arnold and Haymore, 1991; O'Halloran, 1993). Six histidine residues and only one cysteine residue are present in the DtxR polypeptide sequence.

A well conserved putative metal-binding sequence His98-X_3-Cys102-X_3-His106 in a predicted α-helix is present in the central region of DtxR (Fig. 16, p 84). Random mutagenesis demonstrated that amino acid substitutions within this sequence (E100K, W104Q and H106Y) resulted in loss of repressor activity in vivo (Wang *et al.*, 1994). A saturation substitutions study at position Cys-102 with each of the other 19 amino acid residues demonstrated that only the variant DtxR with aspartate at this position retained partial repressor activity (Tao *et al.*, 1993). These findings suggested that the potential metal-chelating histidine and cysteine residues in this domain were essential for the metal-dependent regulatory activity of DtxR. We used site-directed mutagenesis to examine further the roles of His-98, Cys-102, and His-106 in the metal-binding and metal-dependent repressor activities of DtxR.

The plasmid pSKdtxR(-) containing the wild type *dtxR* allele was transformed into *E. coli* K-12 strain CJ236, which contains *dut* and *ung* mutations. The uracil-containing, antisense DNA strand of pSKdtxR(-) was isolated from this transformant with helper

phage R408 (Fig. 18a). This uracil-containing DNA strand was then used as the template for the in vitro synthesis of the complementary strand DNA primed by oligonucleotides containing the desired mutations. The newly synthesized hetero-stranded DNA molecules were analyzed by electrophoresis (Fig. 18b) and transformed into *E. coli* DH5α. Six mutant *dtxR* alleles, H98N, H98R, C102H, C102R, C102S, and H106R, were identified by DNA-sequencing. The system for naming of the variants from site-specific DtxR mutagenesis was the same as that used for naming the variants obtained from random mutagenesis.

2. Repressor activities of site-specific DtxR variants

Each of the site-specific mutant alleles was tested in vivo in *E. coli* DH5α(pCMZ100) for its repressor activity and in *E. coli* DH5α(pCMZ100+pDSK29) for its ability to interfere with wild-type repressor function as described previously (Table 7) (Wang *et al.*, 1994). Amino acid substitutions at positions Cys-102 and His-106 resulted in severe defects in function of DtxR repressor. In contrast, amino acid replacements at position His-98 had little effect on repressor activity.

3. Intracellular production of site-specific mutant proteins of DtxR

To eliminate the possibility that the loss of repressor activity was due to intracellular proteolytic degradation of these DtxR variants, we screened crude cell extracts of *E. coli* strains carrying each mutant allele by immunoblots with rabbit polyclonal antiserum against a DtxR-MelE fusion protein and with a mouse monoclonal

Figure 18. Oligonucleotide-directed site-specific mutagenesis (1.0% agarose gel analysis). **A**) Isolation of single-stranded, uracil containing DNA of phagemid pSKdtxR (-) (antisense strand) from strain CJ236 (*dut* and *ung*). Lane 1 is molecular weight markers. Lane 2 is the control with single-stranded DNA of pSKdtxR (-). Lane 3 is the single-stranded, uracil containing DNA of pSKdtxR (-). Lane 4 is the double-stranded DNA of pSKdtxR (-). **B**) Formation of mutagenized, double-stranded DNA of pSKdtxR. Lane 1 is the single-stranded DNA prep of pSKdtxR (-). Lane 2-7 are the products of in vitro mutagenesis reactions with primers H98, C102R, C102H, C102S, H98, and H106, respectively. Lane 8 is the double-stranded DNA of pSKdtxR (-). **ccc**: covalently closed circular DNA; **sc**: supercoiled DNA.

a. Functional assays for repressor included colonial phenotype and quantitative measurement of *β*-galactosidase (*β*-gal) activity. Increasing colonial color or enzyme activity indicates progressively greater impairment of repressor function as indicated previously (Wang *et al.*, 1994). The control value for *β*-galactosidase activity in *DH5α*(pCMZ100) without a second or third plasmid was 86.0 ±7.1.

b. Increasing colonial color indicates progressively greater inhibition of wild-type DtxR (Wang *et al.*, 1994).

c. Strain designations reflect the amino acid substitution that occurs in the DtxR protein encoded by the *dtxR* allele in the mutant pSKdtxR plasmid.

d. Mutant *dtxR* allele identified by random mutagenesis (Wang *et al.*, 1994)

e. ND = not determined

Table 7. Characterization of Mutant *dtxR* Alleles Isolated from Site-Specific Mutagenesis

STRAIN	FUNCTIONAL ASSAY[a]		DOMINANT NEGATIVE ASSAY[b]
	PHENOTYPE	ENZYME ACTIVITY	
Mutants[c]			
H98N	+	0.8 ±0.1	+/-
H98R	+	1.3 ±0.1	+/-
C102S	+ +	6.0 ±0.2	+ +
C102R	+ + +	11.1 ±1.2	+ +
C102H	+ + +	13.3 ±3.0	+ +
H106R	+ + +	12.3 ±4.5	+ +
H106Y[d]	+ + +	12.0 ±0.3	+
Control			
pSKdtxR	-	0.2 ±0.2	-
pDSK29	-	0.3 ±0.2	ND[e]
pSKlac⁻	+ + +	51.9 ±4.1	ND

anti-DtxR antibody. Expression of these mutant DtxR alleles produced normal amountsof the full length DtxR variants which had the same electrophoretic mobilities as wild- type DtxR (Fig. 19). Cys-102 and His-106 appear to be essential for the normal metalloregulatory function of DtxR, since substitutions for these amino acids abolish repressor function without dramatic changes in the amount of intracellular DtxR protein. In contrast, the His-98 residue is not required for the iron-dependent repressor function of DtxR.

III. Purification of Variant DtxR Proteins by Chromatography on Ni^{2+}-Nitrilotriacetic Acid-Resin

The production of the wild type DtxR from the strong T7 promoter in pKSdtxR (derived from pBluescript KS) or from the strong *lac* promoter in pSKdtxR was low. The overexpression of wild type DtxR was achieved from plasmid pDtxR-7 which contained a modified *dtxR* gene with an optimum distance between the ribosomal binding site and the translational initiation codon (Schmitt and Holmes, 1993). The DNA fragments (*Pst*I/*Sph*I) containing the mutant alleles for H98N, H98R, C102H, C102R, C102S, H106R, and H106Y were prepared and substituted for the corresponding wild type fragment of *dtxR* in pDtxR-7. High yields of these DtxR variants were obtained in *E. coli* DH5α from these newly constructed plasmids. In addition, I found that large amounts of the variants P39L, T40I, and T44I could be produced from appropriate pSKdtxR clones in *E. coli* DH5α induced with IPTG. It is still not clear why there are differences

Figure 19. Western blot analysis of DtxR variants isolated from site-specific mutagenesis. Extracts of *E. coli* DH5α containing pSKdtxR plasmids with wild type or mutant *dtxR* alleles were subjected to 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes, and the DtxR proteins on the membrane were allowed to react with polyclonal rabbit antibody against DtxR-MalE fusion protein. The immobilized antibodies were then allowed to react with an enzyme-labelled second antibody, and the blots were developed using a chromogenic substrate for the enzyme activity (peroxidase). Lane 1 is the control extract from host strain DH5α. Lane 2-9 are the extracts from DH5α stains containing *dtxR* alleles that encode C102R, C102H, C102S, H98N, H98R, H106R, H106Y, and wild type DtxR, respectively. Lane 10 is the molecular weight marker.

of expression from mutant *dtxR* alleles in pSKdtxR. Previous experiments demonstrated that wild type DtxR and the R47H variant bind to the resin and can be purified in a single step by chromatography on Ni^{2+}-NTA-agarose (Schmitt and Holmes, 1993). To determine whether the DtxR variants could bind to the Ni^{2+}-NTA-agarose resin, crude extracts of DH5α(pSKdtxR) containing the wild type or mutant *dtxR* alleles were mixed with Ni^{2+}-NTA-agarose resin at 4°C, and assays for free and bound DtxR were performed by Western blotting (Fig. 20). These experiments showed that each immunoreactive DtxR variant (Fig. 15, p 81) was capable of binding to Ni^{2+}-NTA-agarose resin.

Several variants of DtxR including wild type DtxR were selected for further study and purified by Ni^{2+}-NTA-agarose chromatography as described in Materials and Methods. These included the variants P39L, T40I, T44I and A46V with substitutions in the presumed DNA-binding motif, and the variants isolated from site-specific mutagenesis plus H106Y with substitutions in the presumed metal-binding motif (Fig. 21a & 21b). A sample of R47H purified previously (Schmitt and Holmes, 1993) was also available for analysis.

Wild type DtxR and most of the selected variants eluted from the Ni^{2+}-NTA column at histidine concentrations between 3 and 10 mM. The variant C102H, however, required higher concentrations of histidine (5 to 15 mM) to elute from the column (Fig. 21c). This observation suggested that the variant C102H had a higher affinity to Ni^{2+} than the wild type DtxR.

Figure 20. Binding of immunoreactive mutant DtxR proteins to Ni^{2+}-NTA-agarose. *E. coli* DH5α containing wild type or mutant *dtxR* alleles were lysed by sonication, and 15 μl samples of the crude cell extract were mixed with 30 μl samples of Ni^{2+}-NTA-agarose resin at 4°C for 1 hour. After centrifugation at 5000 rpm for 1 min, 10 μl of supernatant was removed (these fractions contained proteins that did not bind to the Ni^{2+} resin, designated [F]). The resin was washed 3x with 1.0 ml samples of sonication buffer. The resin was then resuspended in 15 μl of sonication buffer, and 10 μl of the resuspended resin was removed (these fractions contained proteins that bound to the Ni^{2+} resin, designated [B]). Protein samples were subjected to 10% SDS-PAGE, and DtxR was identified by Western blot analysis using rabbit polyclonal anti-DtxR antibodies.

Figure 21. Protein purification by Ni^{2+}-NTA-agarose column. The cell cultures were resuspend in 3 ml samples of sonication buffer (10 mM phosphate buffer pH 7.0, 50 mM NaCl) and lysed by sonication. 2.5 ml samples of crude cell extract were loaded on 1.0 ml Ni^{2+}-NTA-agarose columns. DtxR was eluted from each column by a step gradient of histidine in sonication buffer. 10 μl samples of fractions eluted from the column were analyzed by 10% SDS-PAGE. Panels A., B., and C. show the purification of wild type DtxR, variant P39L, and variant C102H, respectively. Lane 1 of panel A. and lane 2 of panels B. and C. are crude cell extracts of DtxR. Lane 2 of A. and Lane 3 of B. and C. are samples of the crude cell extract passing through the column. Lane 3 of A. and lane 4 of B. and C. are the wash of the column. Lane 5-13 of A. and lane 6-14 of B. and C. are eluted fractions of the step gradient of histidine at concentrations of 1, 2, 3, 5, 7.5, 10, 15, 20, 25, and 50mM. Lane 14 of A. and lane 1 of B. and C. are molecular weight markers. Proteins were detected by Coomassie blue (G250) staining.

IV. In Vitro Functional Analysis of Purified DtxR Variants

From random mutagenesis and polypeptide sequence analysis, two categories of DtxR variants were identified with amino acid substitutions in the putative DNA-binding domain and in the putative metal-binding domain. Since DtxR belongs to the metalloregulatory protein family, which can relay a transition metal signal into gene expression, binding of DtxR to the *tox* operator is dependent on binding of DtxR to divalent cations. Therefore, DtxR variants with impaired metal-binding activity are also likely to exhibit decreased DNA-binding activity. In contrast, variants which can not bind to the *tox* operator may have intact metal-binding activity. The purified wild type and variant DtxR proteins were therefore subjected to direct assays for DNA-binding activity and metal-binding activity.

1. Metal-binding activities of DtxR and its variants

i. $^{63}Ni^{2+}$-binding assays of wild-type DtxR

Some of the first-row transition divalent cations, such as Fe^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, but not Cu^{2+} or Fe^{3+} can function as corepressors for DtxR *in vitro* (Schmitt *et al*, 1992; Schmitt and Holmes, 1993 & 1994; Tao *et al.*, 1992a & 1992b). Binding of divalent cations by the DtxR aporepressor is presumed to cause an allosteric change in the conformation of DtxR, which enables the activated repressor to bind to the *tox* operator. I used $^{63}Ni^{2+}$ as a convenient and suitable isotope for development of a quantitative assay for the metal-binding activity of DtxR, based on a published method for Hg^{2+}-binding activity of the MerR protein (Shewchuk *et al.*, 1989a & 1989b). The

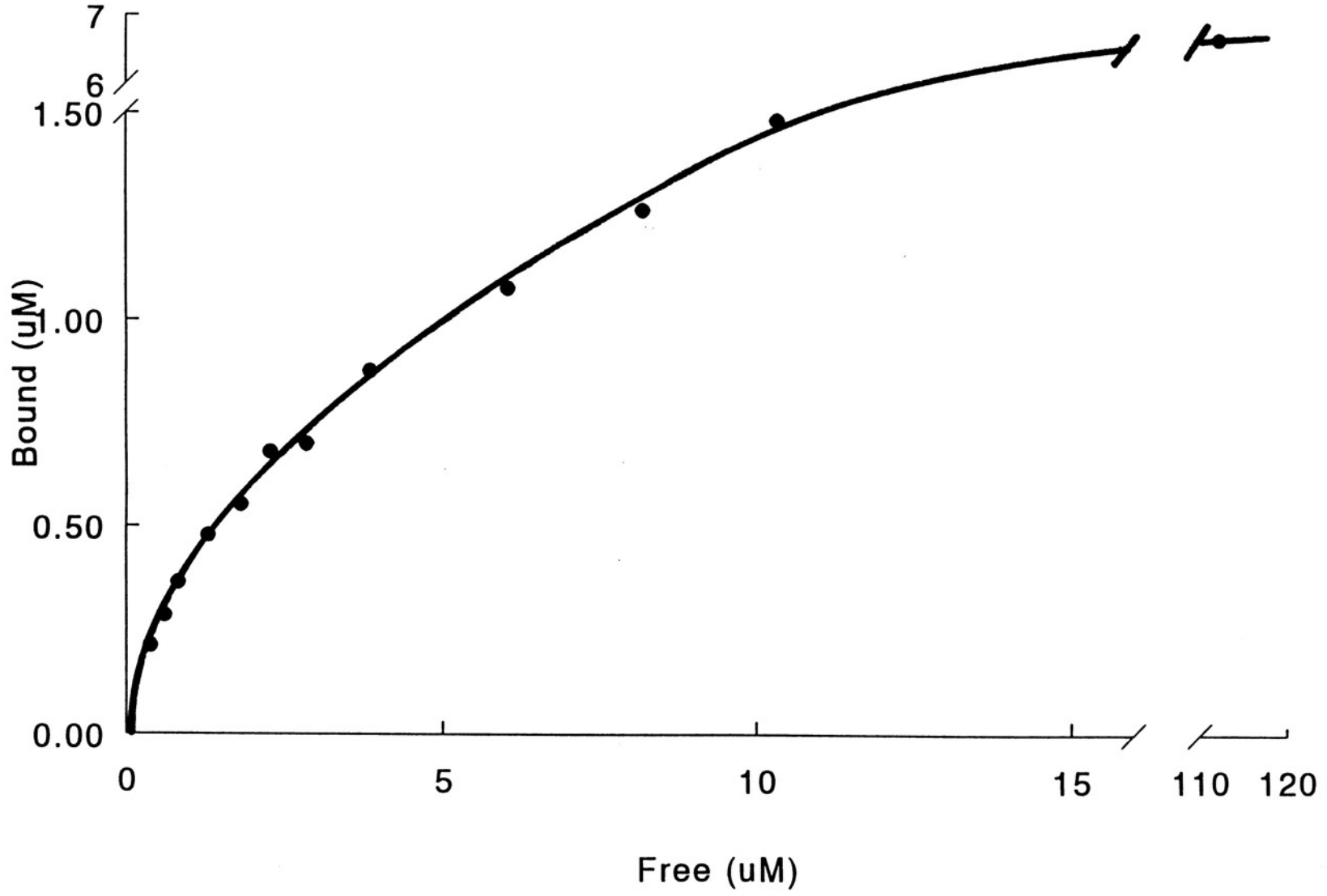
saturation binding data for Ni^{2+}-binding by wild type DtxR is shown in Fig. 22a, and the Scatchard transformation of these binding data is shown in Fig. 22b. Data analysis and curve-fitting for ligand binding of DtxR were performed by using the software program LIGAND (NIH, Bethesda, MD). The Ni^{2+}-binding data for wild type DtxR fit to a model with two classes of Ni^{2+} specific binding sites (high affinity, and low affinity) plus nonspecific binding sites. The equilibrium dissociation constant (K_d) for the high affinity site was 0.98×10^{-6} M. Maximum binding at the high affinity site was estimated to be 0.8 moles of Ni^{2+} per mole of DtxR monomer, consistent with one high affinity Ni^{2}-binding site per monomer. Since there were few points of the experimental data corresponding with the low affinity binding phase, the equilibrium dissociation constant (K_d) and maximum binding for the low affinity site could not be accurately determined. Results from protein crosslinking assays and HPLC chromatography demonstrated that DtxR protein existed in a dimeric form both in the presence and in the absence of divalent cations (Zhang *et al.*, personal communication). Therefore, there should be two high affinity Ni^{2+}-binding sites plus low affinity Ni^{2+}-binding sites per DtxR dimer.

ii. Competitive $^{63}Ni^{2+}$-binding assays of wild-type DtxR

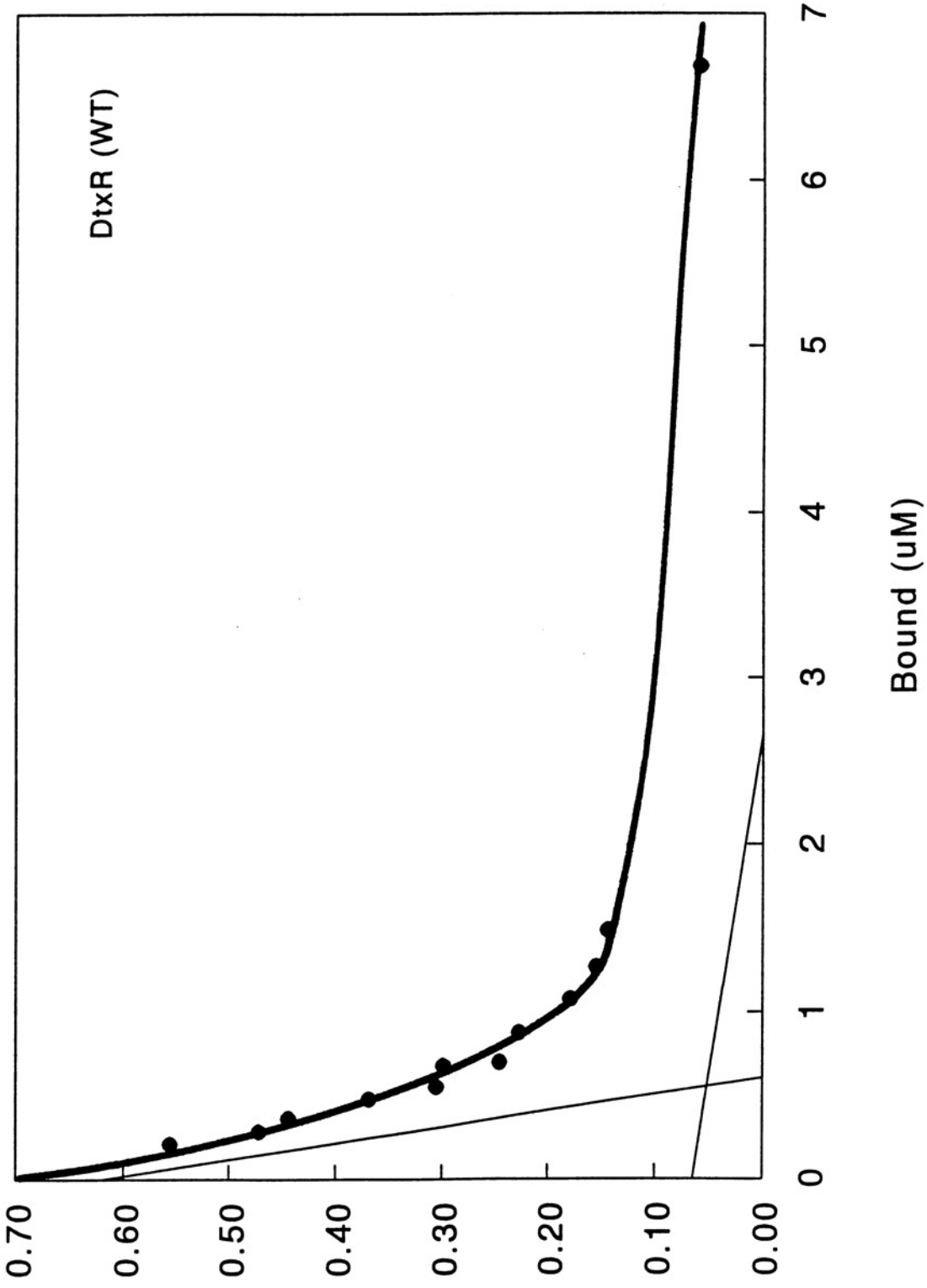
To determine if various metal ions could compete with $^{63}Ni^{2+}$ for binding to DtxR, I performed a series of competitive $^{63}Ni^{2+}$-binding assays. For each reaction, a nonradioactive divalent cation was present at 100 fold excess over the concentration of $^{63}Ni^{2+}$in a standard $^{63}Ni^{2+}$-binding assay. Inhibition of the $^{63}Ni^{2+}$-binding to DtxR in these experiments by excess Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} indicated that all of

Figure 22. Binding of $^{63}Ni^{2+}$ by wild type DtxR. DtxR at a final concentration of 20 μg/ml (equivalent to monomer at 0.79 μM in 250 μl reaction mixture was incubated with $^{63}Ni^{2+}$ at molar ratios of Ni^{2+} to DtxR from 0.5 to 30. Bound and free $^{63}Ni^{2+}$ were then separated by filtering the samples through Immobilon™ membranes as described in *Materials & Methods*. Panel **A)** shows the saturation curve for binding of $^{63}Ni^{2+}$ by DtxR. Panel **B)** shows the Scatchard transformation of the binding data. Each point represents the mean of three independent determinations. The standard deviations did not exceed 10 percent of the mean values. The data analysis and curve-fitting for Ni^{2+}-binding were performed by using software program LIGAND (NIH, Bethesda, MD). Solid curve is computerized best fit for two classes of Ni^{2+}-specific binding sites plus non-specific binding model. The two binding components are indicated by the solid straight lines.

A



B


DtxR (WT)
Bound / Free
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
1
2
3
4
5
6
7
Bound (uM)

these divalent cations competed for the same metal-binding site of DtxR (Fig. 23). From these results, the apparent rank ordery of binding affinity of these divalent cations to DtxR is $Fe^{2+} \approx Cu^{2+} \approx Mn^{2+} > Ni^{2+} > Cd^{2+} \approx Zn^{2+} > Co^{2+}$. The fact that excessive Fe^{3+} molecules failed to inhibit $^{63}Ni^{2+}$-binding was consistent with the previous finding that Fe^{3+} could not activate repressor function.

iii. Variants with amino acid replacements in the metal-binding domain

The Ni^{2+}-binding activity of purified wild-type DtxR, H98N, H98R, C102H, C102R, C102S, H106R, and H106Y was compared at concentrations of Ni^{2+}corresponding approximately with 20%, 40%, and 60% calculated occupancy of the Ni^{2+}-binding site of wild-type DtxR. More complete binding studies were performed and were analyzed for selected DtxR variants.

(1) Variants with His-98 and His-106 substitution The DtxR variants with amino acid substitutions at His-98 or His-106 displayed a decrease in Ni^{2+}-binding, to about 50% of the level observed with wild-type DtxR (Fig. 24). None of these variants showed a complete loss of Ni^{2+}-binding activity. Scatchard transformations of the binding data for the H98R and H106R variants are shown Figure 25a and 25b. Analysis of ligand binding with the program LIGAND revealed that the binding data for H98R fit best to a model with one class of high-affinity Ni^{2+}-specific binding site plus nonspecific sites, and the data for H106R fit best to a model with two classes of Ni^{2+}-specific binding sites (high-affinity and low affinity) plus nonspecific sites. The slopes of the plots for the

Figure 23. Competitive binding of $^{63}Ni^{2+}$ to wild type DtxR by other heavy metal molecules. Reaction mixtures contained 20 μg of DtxR/ml (equivalent to monomer at 0.79 μM), 0.592 μM $^{63}Ni^{2+}$, and 59.2 μM of a nonradioactive metal ion as shown. Bound and free $^{63}Ni^{2+}$ were then separated by filtering the samples through Immobilon™ membranes as described in *Materials & Methods*. Each point represents the mean of four independent determinations.

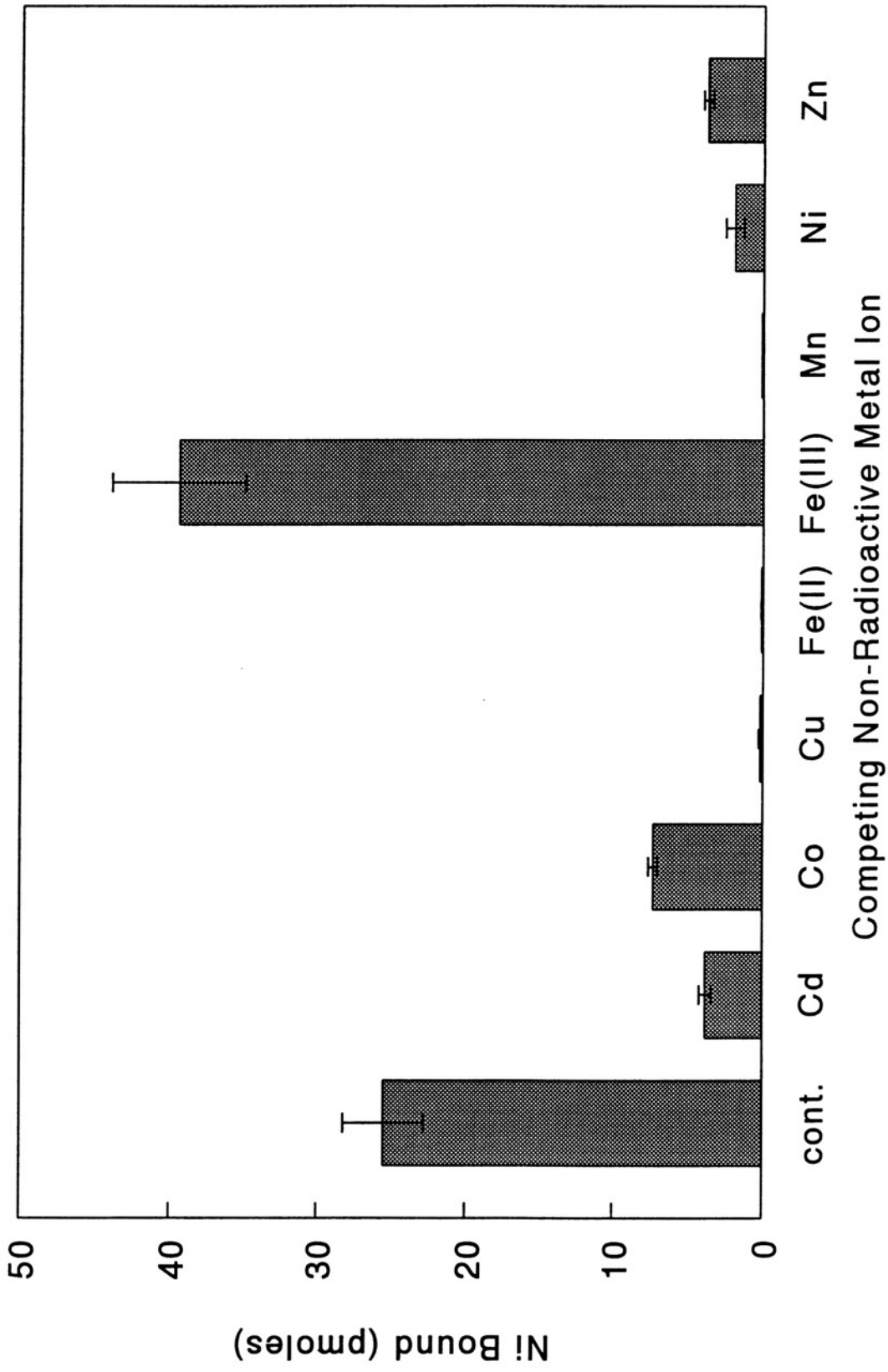

Ni Bound (pmoles)
0
10
20
30
40
50
cont.
Cd
Co
Cu
Fe(II)
Fe(III)
Mn
Ni
Zn
Competing Non-Radioactive Metal Ion

Figure 24. Binding of $^{63}Ni^{2+}$ by wild type DtxR and variants with amino acid substitutions in the metal-binding domain. Samples containing wild type or mutant DtxR protein at 17 μg/ml (equivalent to monomer at 0.66 μM) were incubated with $^{63}Ni^{2+}$ at 0.78, 1.96 or 4.19 μM and then processed by filtration through Immobilon™ membranes as described in *Materials & Methods*. Each value represents the mean of four independent experiments, and error bars indicate ± one standard deviation.

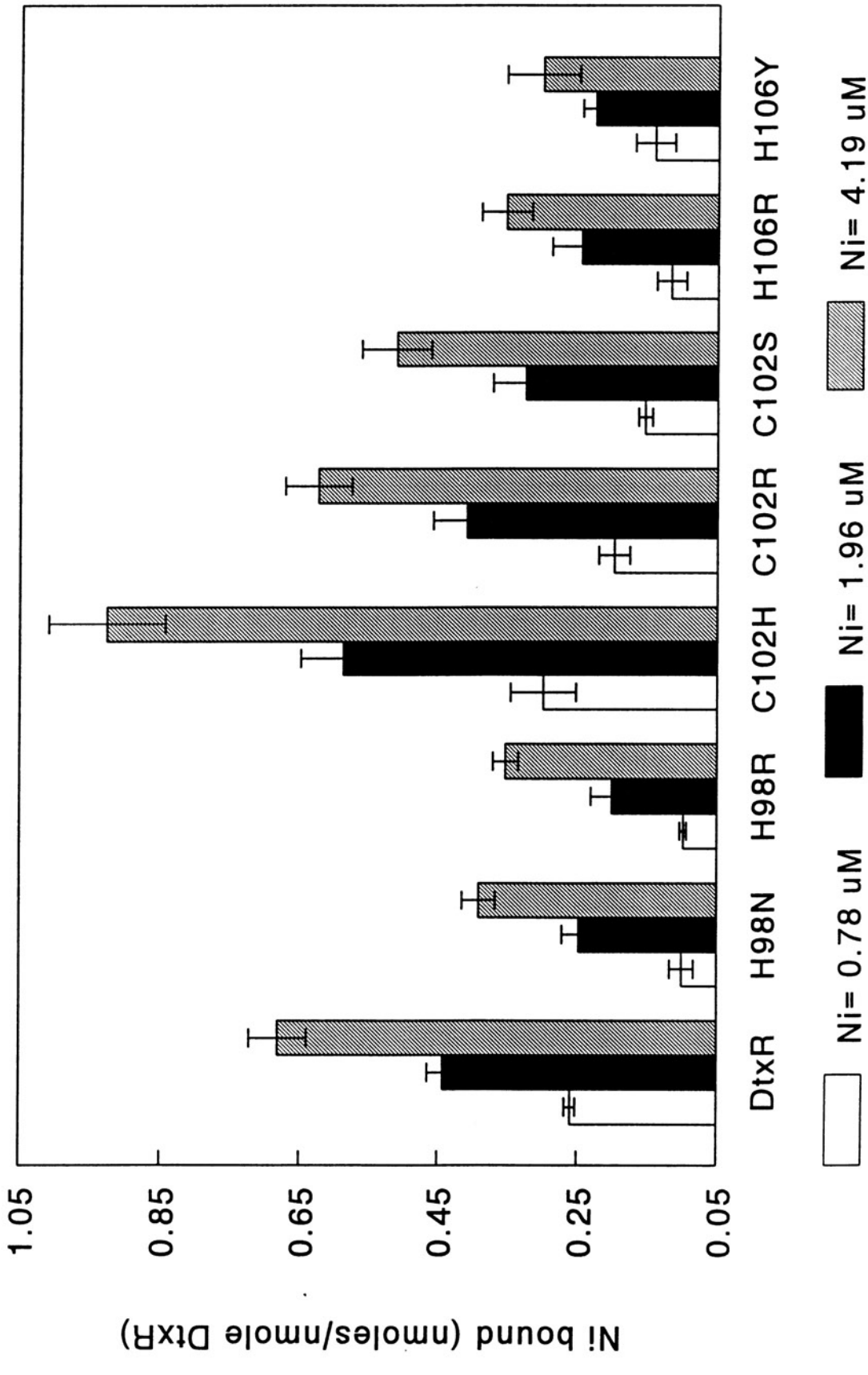
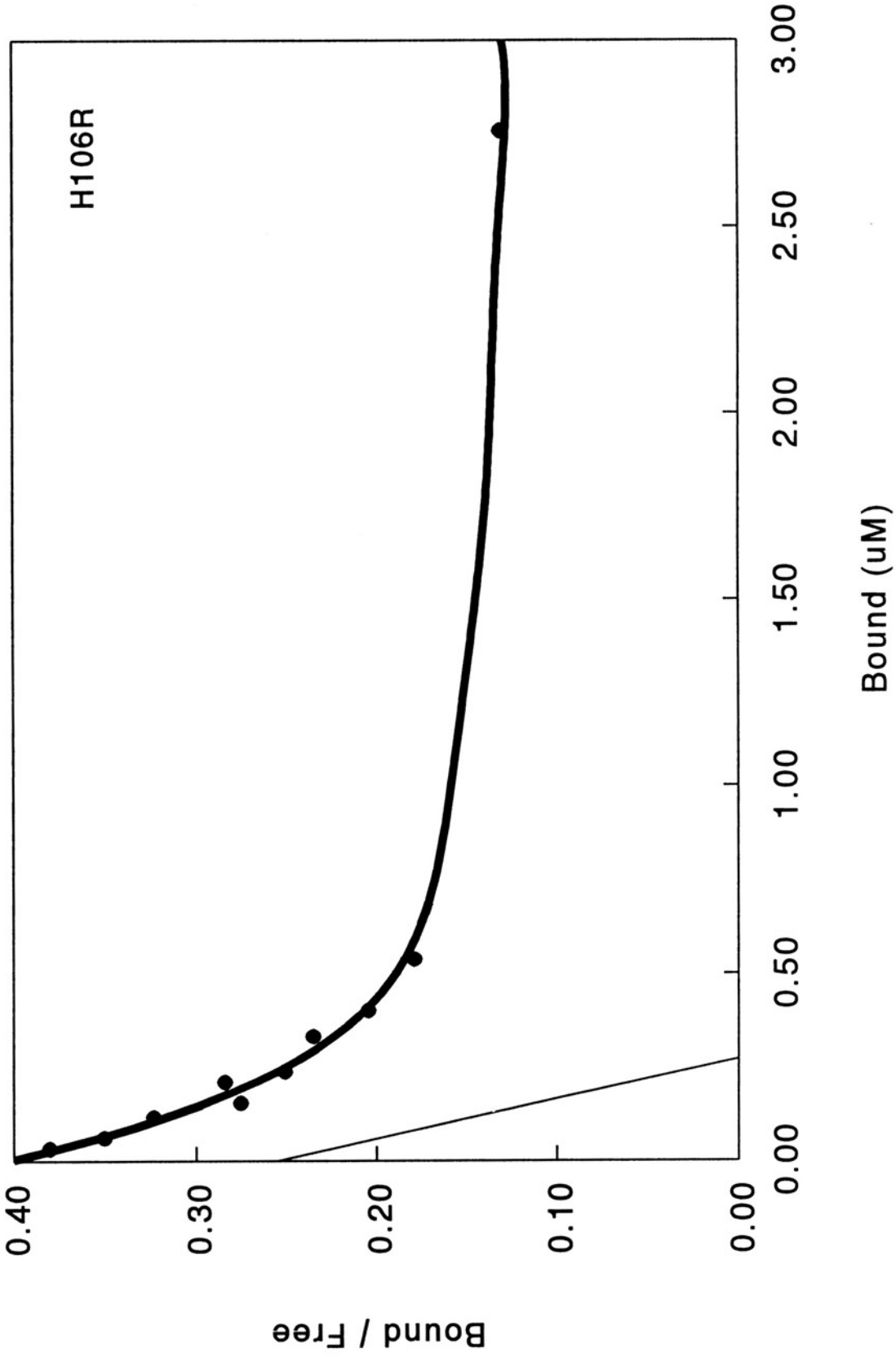
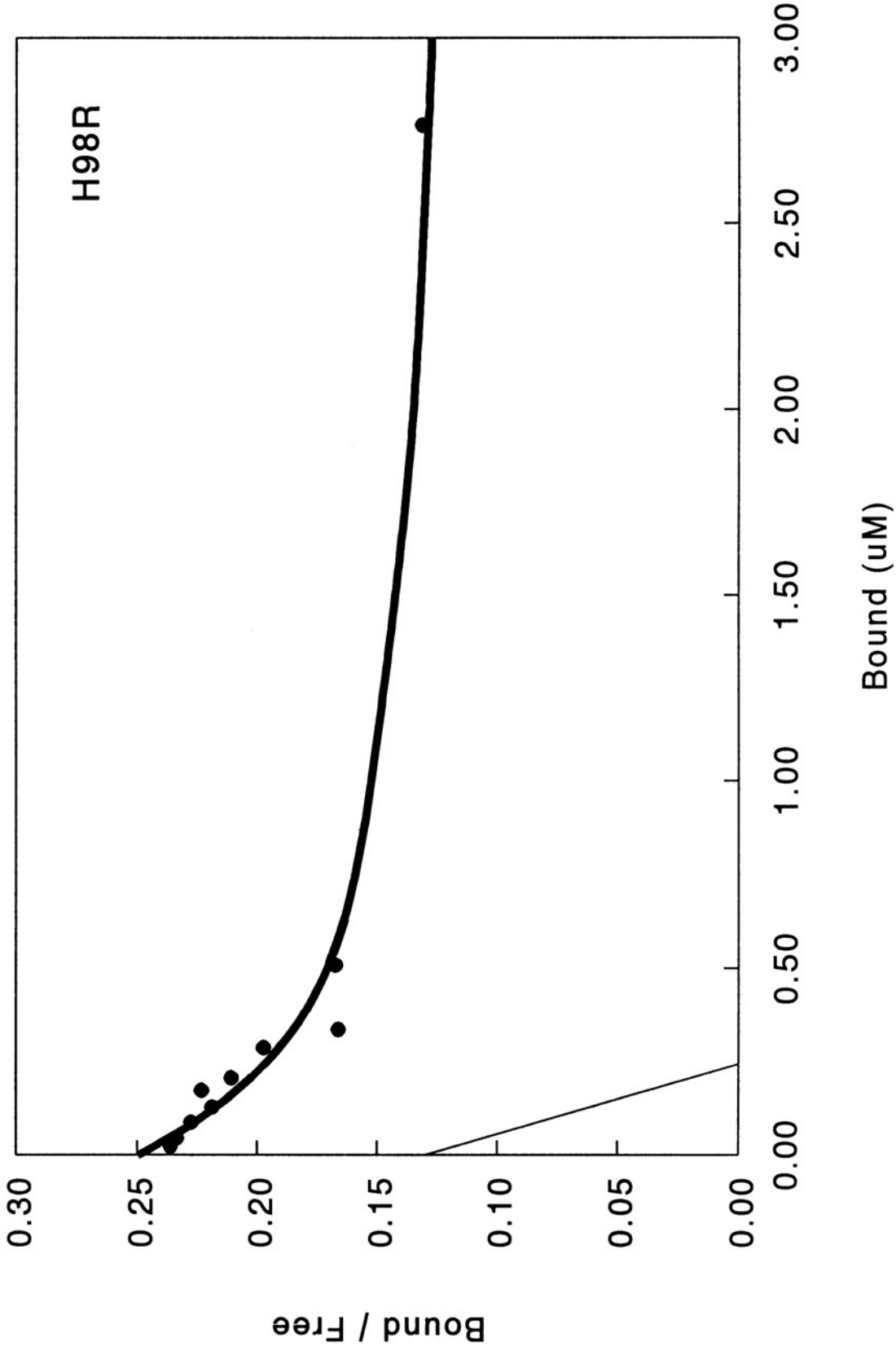

Ni bound (moles/mole DtxR)
1.05
0.85
0.65
0.45
0.25
0.05
DtxR
H98N
H98R
C102H
C102R
C102S
H106R
H106Y
Ni= 0.78 uM
Ni= 1.96 uM
Ni= 4.19 uM

Figure 25. Scatchard transformation analysis of $^{63}Ni^{2+}$-binding of wild type DtxR and variants H98R and H106R. The data analysis and curve-fitting for Ni^{2+}-binding were performed by using software program LIGAND (NIH, Bethesda, MD). The best fit for the data was obtained using the one binding site model for H98R and the two binding-site model for H106R. The solid line is the computerized best fit for the single binding site of H98R and the high-affinity binding site for H106R. A best fit binding for the low-affinity site of H106R is not show because it could not be determined accurately from the available data.


H106R
Bound / Free
0.40
0.30
0.20
0.10
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
Bound (uM)


H98R
Bound / Free
Bound (uM)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00

H98R and H106R variants were similar to that for DtxR, and their calculated equilibrium dissociation constants were 1.8 x 10^{-6} M and 0.94 x 10^{-6} M, respectively. The maximum high-affinity binding for these variants (0.33 and 0.34 moles of Ni^{2+}/ mole of DtxR, respectively) was approximately halfof the value for wild-type DtxR. Because there was not enough experimental data in the appropriate range of concentrations for the low-affinity binding sites, we could not compare the low affinity binding of wild type DtxR with that of the variants H98R and H106R. These findings, together with earlier observations, suggested that substitution for one histidine residue at either position 98 or position 106 of DtxR polypeptide eliminated one of two similar high-affinity Ni^{2+}-binding sites in the DtxR dimer. Furthermore, they suggested that His-98 and His-106 might be located in two separate, high-affinity, Ni^{2+}-binding sites of DtxR.

(2) Variants with Cys-102 substitutions Variants of DtxR with amino acid replacements at position Cys-102 behaved differently from those at His-98 and His-106 in the Ni^{2+}-binding assay. The variants with favored or unfavored metal-coordinating ligand substitutions at Cys-102 also differed from on another (Fig. 24, p 113). C102R did not differ significantly from wild type DtxR in the Ni^{2+}-binding assay. In contrast, C102H exhibited a somewhat higher binding affinity to Ni^{2+}, and C102S showed a somewhat lower binding affinity to Ni^{2+}. These findings indicated that Cys-102 is not essential for the metal-binding activity of DtxR. Nevertheless, cysteine at position 102 is essential for the repressor activity of DtxR (Tao *et al.*, 1993; and this study). Taken together, these findings suggest that coordination of divalent metal ions by cysteine at

position 102 is important for signal transduction by DtxR as a metalloregulatory protein but is not required for metal binding activity.

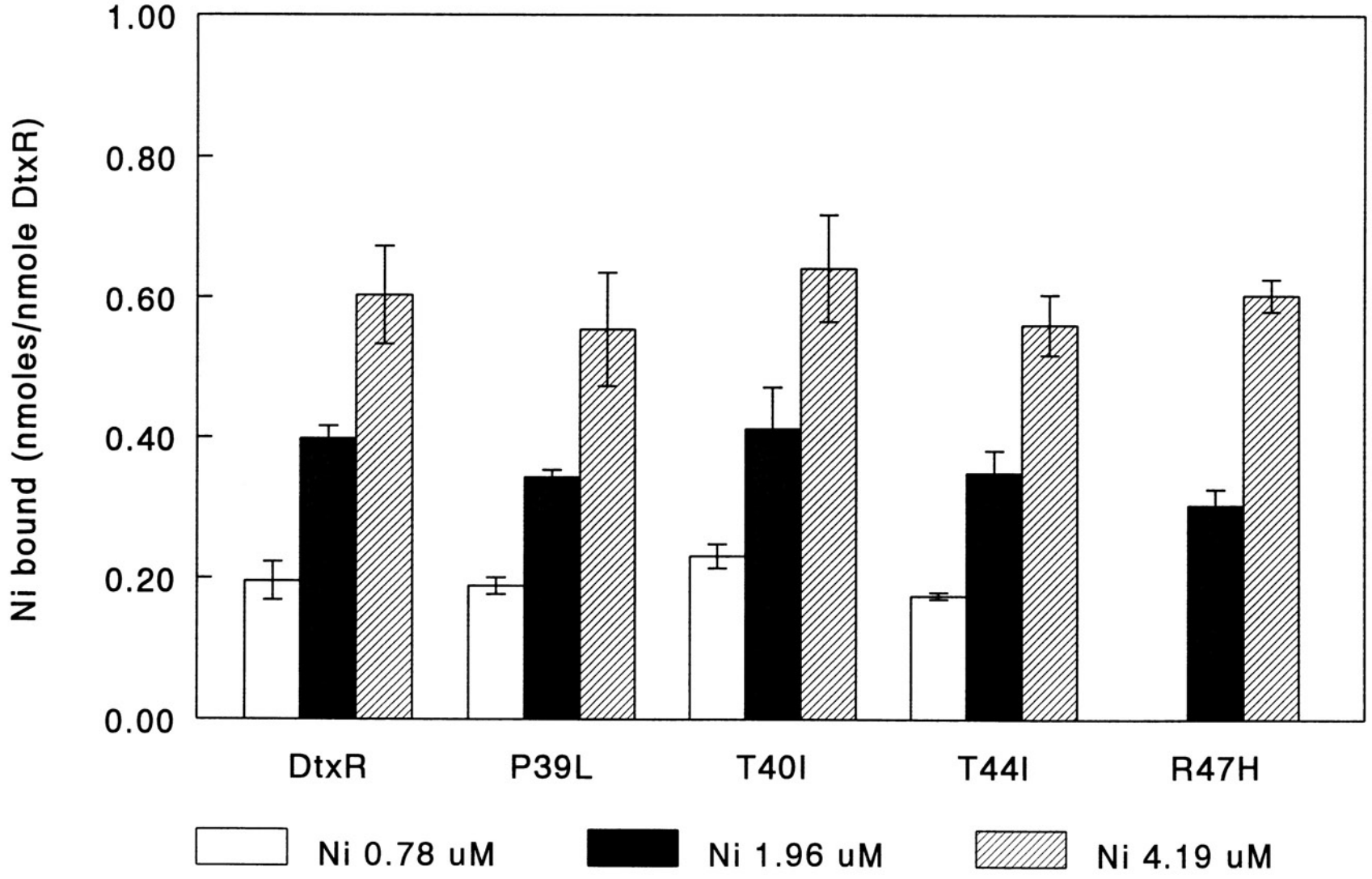
iv. Variants with substitutions in the putative DNA-binding domain

The Ni^{2+}-binding activities of purified wild type DtxR, P39L, T40I, T44I, and R47H were compared at concentrations of Ni^{2+} corresponding approximately with 20%, 40% and 60% calculated occupancy of the Ni^{2+}-binding sites of wild type DtxR (Fig. 26). At the higher and lower Ni^{2+} concentrations, the Ni^{2+}-binding activities of P39L, T40I, T44I, and R47H, which have substitutions within the presumed DNA-binding region, were not significantly different from that of wild type DtxR. At the intermediate Ni^{2+} concentration, the P39L and R47H variants showed a slight but statistically significant ($P < 0.05$) decrease in Ni^{2+}-binding. These findings indicate that substitutions within the DNA-binding domain did not dramatically affect the metal-binding activity of DtxR and support the model that DtxR has separate DNA-binding and metal-binding domains.

3. DNA-binding activities of DtxR and its variants

The sequence specific DNA-binding activities of wild-type and variant DtxR proteins were compared by gel mobility shift assays and DNase I protection analysis in the presence of various divalent cations. The results of these experiments are summarized in Table 8.

Figure 26. Binding of $^{63}Ni^{2+}$ by wild type DtxR and variants with amino acid substitutions within DNA-binding domain. Samples containing wild type or mutant DtxR protein at 85 μg/ml (equivalent to monomer at 3.3 μM) were incubated with $^{63}Ni^{2+}$ at 0.78, 1.96 or 4.19 μM and then processed by filtration through Immobilon™ membranes as described in *Materials & Methods*. Each value represents the mean of four independent experiments, and error bars indicate ± one standard deviation.


1.00
0.80
0.60
0.40
0.20
0.00
Ni bound (nmoles/nmole DtxR)
DtxR
P39L
T40I
T44I
R47H
Ni 0.78 uM
Ni 1.96 uM
Ni 4.19 uM

a. Gel mobility shift assays were performed as described in Materials and Methods. +, significant shift; +/-, minor shift; -, no shift.

b. DNase I protection reactions were performed as described earlier with divalent cations as indicated at 150 μM (Schmitt *et al.*, 1992). + indicates full protection; +/- partial protection; - no protection.

Table 8. DNA-Binding Assay with DtxR Variants

DtxR	Gel Shift[a]	DNase I Protecction with[b]						
	Co^{2+}	Fe^{2+}	Co^{2+}	Zn^{2+}	Ni^{2+}	Cd^{2+}	Mn^{2+}	Cu^{2+}
WT DtxR	+	+	+	+	+	+	+	-
P39L	-	-	-	-	-	-	-	-
T40I	-	-	-	-	-	-	-	-
T44I	-	-	+	-	+	-	-	-
H98N	+/-	+	+	+	+	+	+	-
H98R	+/-	+/-	+	-	+	+	+/-	-
C102H	+	-	+	+	-	+	-	-
C102R	-	-	-	-	-	-	-	-
C102S	+	-	+	+/-	+	+/-	+/-	-
H106R	-	-	-	-	-	-	-	-
H106Y	-	-	-	-	-	-	-	-

i. Wild type DtxR in DNA-binding assays

At the concentration of 150 μM, divalent cations Fe^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, but not Cu^{2+}, enabled wild-type DtxR to bind to a DNA fragment carrying the *tox* promoter-operator region (Fig. 27). A major binding region on the DNA fragment protected by wild type DtxR, containing a 19-bp consensus DtxR-binding box, was identical to the protection region described by Schmitt (1992). In the presence of Co^{2+} or Ni^{2+}, wild-type DtxR was able to bind to a second protection region, which was a 28 bp sequence containing a 5 bp inverted repeat sequence separated by a 10 bp sequence (Fig. 27). This second protection region was located upstream from the putative -35 region of the *tox* promoter (position -129 to -101 from the translational start codon). The significance of this second protection region in repressor function of DtxR is unknown.

ii. Variants with substitutions within the putative DNA-binding Domain

Amino acid alterations within the DNA-binding domain (variants P39L, T40I, T44I) had a tremendous effect on DNA-binding. All of the tested divalent cations failed to activate the variants P39L and T40I to protect the *tox* operator from DNase I digestion. Only Co^{2+} and Ni^{2+} activated the T44I variant in the same protection assay. The variant T44I, like the previously characterized variant R47H (Schmitt and Holmes, 1993), bound to the major protection region in the *tox* operator but not to the second region when activated by the cofactor Co^{2+} or Ni^{2+}.

Figure 27 DNase I protection assays with wild-type and variant DtxR proteins. Various divalent cations were present at concentrations of 150 μM in reaction mixtures. A+G dideoxy sequencing ladder for adenine and guanine nucleotides, respectively (Panel A), was prepared by Maxam-Gilbert DNA sequencing Kit (SIGMA Chemical Company, St. louis, MO). DNase I reactions were performed as described earlier (Schmitt *et al.*, 1992). The left - is the negative control reaction without any DtxR protein. The right - is the binding reaction without any divalent cation.

iii. Variants with alterations within metal-binding domain

(1) Substitutions at position His98 and His106 Replacements at position His-98 did not cause big changes in the DNA-binding activity of DtxR. The metal-dependent protection pattern in DNase I protection assays with the variant H98N was identical to that of wild-type DtxR (Table 8, p 122). The protection pattern with the variant H98R differed from that of wild type DtxR, exhibiting no protection with Zn^{2+} and weaker protection with Fe^{2+} or Mn^{2+} used as the cofactor (Table 8, p 122). These observations are consistent with the results of in vivo biological assays (Table 5, p 75; Table 7, p 93), which indicated that the His-98 residue was not essential for the metalloregulatory function of DtxR. In contrast, residue His-106 was vital for repressor function, since both amino acid substitutions at this position, H106R and H106Y, completely abolished the DNA-binding activity of DtxR in these DNase I protection experiments (Table 8, p 122).

(2) Substitutions at position Cys-102 Similar to the observations in $^{63}Ni^{2+}$-binding assays, the DtxR variants with different substitutions for residue Cys-102 behaved differently in DNA-binding assays, although all were inactive with the physiological cofactor Fe^{2+}. None of the divalent cations tested activated the C102R variant to bind to the *tox* operator. The variant C102H, which exhibited a higher binding activity for $^{63}Ni^{2+}$ than wild-type DtxR, and the variant C102S, with lower binding activity for $^{63}Ni^{2+}$, responded differently to activation by specific divalent cations. Co^{2+} was a functional cofactor both for C102H and C102S. Cd^{2+} and Zn^{2+} fully activated the C102H variant to protect the *tox* operator from DNase I digestion, but they only partially activated the

C102S variant. Mn^{2+} and Ni^{2+}, on the contrary, failed to activate the C102H variant in the DNase I protection assays, but they fully or partially activated the C102S variant. We performed additional DNase I protection assays to compare wild-type and the C102H variant with respect to the concentrations of Co^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} required to activate their DNA-binding activities. The lowest concentrations of Co^{2+} and Zn^{2+} required for activating DNA-binding of wild-type DtxR and the C102H variant were the same (50 μm for Co^{2+}, and 7.5 μM for Zn^{2+}), but the lowest concentration of Fe^{2+} needed to activate the variant C102H (2 mM) was at least 10 fold higher than that needed for wild-type DtxR (150 μM) (data not shown). Overall, these observations confirmed the finding that residue Cys-102 in the metal-binding site is essential for the repressor activity of DtxR (Tao *et al.*, 1993) and established that substitution of particular amino acids for Cys-102 can cause dissociation between binding activity of DtxR for specific divalent cations and the activation of DNA-binding activity which is the normal consequence of metal-binding by DtxR in its physiological role as a metalloregulatory protein.

DISCUSSION

I. Distribution of Random Amino Acid Substitutions Within DtxR and Polypeptide Sequence Analysis

DtxR is an iron-dependent repressor in the Gram positive bacterium *C. diphtheriae*. It differs in DNA-binding specificity from the Fur protein, its counterpart in Gram negative bacteria (Bagg and Neilands, 1987; Schmitt and Holmes, 1993 & 1994). The structural basis for the biological activity of DtxR has not yet been defined in detail. In this dissertation, I introduced random mutations into the 5′, middle, and 3′ segments of the *dtxR* gene by *in vitro* bisulfite mutagenesis and characterized a representative set of clones with decreased DtxR activity.

Clones with diminished repressor activity represented a greater proportion of the mutagenized pool when bisulfite treatment was targeted to the 5′ segment or middle segment of *dtxR*. Among 102 mutant alleles sequenced, including 34 that had been mutagenized in each third of the *dtxR* gene, I identified 18 with a single missense mutation, 2 with a single chain-terminating mutation, and 82 with multiple mutations or duplications of mutations already identified. Strikingly, all but one of the single amino acid substitutions that impaired repressor activity were in the amino-terminal half of DtxR (Fig. 16, p 84), and most of the sequenced repressor-deficient mutants isolated from C-terminal fragment mutagenesis contained multiple mutations in *dtxR*. Boyd & Murphy (1992) reported that the wild type DtxRs from *C. diphtheriae* strains 1030 and C7 differ by six amino acid residues, all of which are located in the carboxyl-terminal third of the

molecule. Taken together, these findings indicate that the amino-terminal half of DtxR is particularly important for its biological function.

The single amino acid substitutions that severely diminished or abolished repressor activity (giving +, ++, or +++ phenotypes in Table 5, p 75) were not randomly distributed in the amino-terminal half of DtxR. Instead, they were clustered in three distinct regions that appear to be important for different functions of DtxR.

Six mutant proteins (P39L, T40I, T44I, A46V, R47H, and G52E) had amino acid substitutions between Pro39 and Gly52 in the distal part of a predicted helix-turn-helix motif (Fig. 16, p 84). This sequence exhibits homology with the DNA-recognition region of the LacR repressor and, to a lesser extent, with several other well characterized repressors (Fig. 17, p 86) (Brennan and Matthews, 1989; Harrison, 1991; Kolkhof *et al.*, 1992; Pabo and Sauer, 1992). We concluded that the region from Pro39 to Gly52 in DtxR is likely to be involved in recognition of the *tox* operator and other DtxR-regulated operators of *C. diphtheriae*. Based on secondary structural predictions, the proposed hinge region between two helixes contains five amino acid residues, which is similar to the hinge region of the LexA protein, and is considered to be relatively larger than those of other DNA-binding proteins, such as Cap, λ Cro, etc. Two amino acid substitutions P39L and T40I, which produce relatively inactive proteins and strongly interfere with wild type DtxR repressor function, were located within this hinge region. Therefore, a relatively large hinge region in the DNA-binding domain in DtxR may be essential to maintain the correct orientation of the DNA recognition helix in the DNA-repressor complex.

A high-affinity chelating site for a divalent cation can be formed from as few as two properly positioned metal-coordinating ligands in a protein. Histidines or cysteines are important metal-coordinating ligands at neutral pH, and two histidines or cysteines separated by three intervening residues in an α-helix is a potential metal-binding motif (Arnold and Haymore, 1991; Ryden, 1989; Saito *et al.*, 1991). The His98-XXX-Cys102-XXX-His106 sequence in DtxR fits this pattern, and previous reports implicated Cys102, the only cysteine residue, in the metal binding activity of DtxR (Tao *et al.*, 1993). Three mutant proteins (E100K, W104Q, and H106Y) had amino acid substitutions within this sequence. I concluded that the sequence from His98 to His106 is the probable binding site for divalent cations that activate DtxR.

A third cluster of substitutions (including A72V, R77H, R84H, and D88N) was within a predicted α-helix from residues Thr70 to Gly90 (Fig. 16, p 84). This sequence is located between the presumed DNA-recognition region and the presumed metal-binding site, and we speculate that it is involved in conformational changes induced by metal binding that activate or expose the DNA-recognition domain of DtxR.

Direct evidence from protein crosslinking analysis and HPLC chromatography (S. Zhang and R. K. Holmes, personal communication) demonstrated that wild type DtxR exists in dimeric form with or without divalent cations in the buffer. Dominant-negative phenotypes are often observed with mutant forms of polypeptides from oligomeric proteins that lose biological activity but retain the ability to form nonfunctional oligomers. As shown in Fig. 16 (p 84), variants with the dominant negative phenotype were found in all three clusters of substitutions. This observation suggested that the monomers of

DtxR variants containing amino acid alternations within these three regions could form heterodimers with the wild type DtxR subunit. Thus, it is unlikely, therefore, that these three regions comprise the dimer-forming domain of DtxR, or at least, they do not appear to play a major role in dimer formation. Additional studies will be needed, however, to develop an assay to distinguish between monomeric and dimeric forms of DtxR in crude bacterial extracts and to demonstrate directly whether any of our variant forms of DtxR are deficient in the ability to assemble into dimers.

II. DNA-Binding Domain of DtxR

Previous studies showed that the binding of DtxR to the *tox* operator was dependent on the binding of divalent cations (Schmitt *et al.*, 1992; Tao *et al.*, 1992). In metalloregulation of DtxR, it is clear that the signal is relayed from metal-binding to DNA-binding. Therefore, a mutation in the *dtxR* gene that causes a defect in DNA-binding will not necessarily affect the metal-binding activity of DtxR. On the contrary, a mutation that destroys metal-binding activity is likely to interfere with expression the DNA-binding activity of DtxR. The purified variants P39L, T40I, and T44I, with amino acid substitutions within the putative DNA-binding domain were subjected to in vitro DNA-binding analysis and metal-binding analysis. $^{63}Ni^{2+}$-binding assays exhibited that all these variants maintained the wild type level of metal-binding activity. In gel mobility shift assays, these variants all lacked Co^{2+} activated, sequence-specific DNA-binding activity. DNase I protection experiments revealed that the variants P39L and T40I with amino acid substitutions in the predicted hinge region of the DNA-binding domain could

not bind to the *tox* operator in the presence of any divalent cation. The variant T44I, however, was activated by Co^{2+} or Ni^{2+} but not by Fe^{2+}, Cd^{2+}, Mn^{2+}, and Zn^{2+} in DNase I protection assays. These findings demonstrated directly that the binding activity of P39L, T40I, and T44I for the *tox* operator is significantly less than that of wild type DtxR. They provided an explanation for the decreased repressor activity of these variants in vivo in the *E. coli* reporter gene assay system on the basis of loss of specific DNA-binding activity rather than loss of metal-binding activity. They also support the proposal that the predicted hinge region of the DNA-binding domain of DtxR is essential to maintain the sequence-specific DNA-binding activity of the repressor complex.

In a previous study (Schmitt and Holmes, 1993), R47H exhibited DNA-binding activity similar to that of variant T44I in a DNase I footprinting assay, but only at higher concentrations of metal ions than are needed for the DNA-binding activity of wild type DtxR. Therefore, R47H was presumed to have decreased binding activity for divalent cations. The subsequent direct demonstration in the $^{63}Ni^{2+}$-binding assay that binding of Ni^{2+} by T44I and R47H and wild type DtxR are similar (Fig. 26, p 120) contradicted this hypothesis. These results suggested that activation of repressor activity is less tightly coupled to binding of divalent cations for T44I and R47H than it is for wild type DtxR. Substitutions within the DNA recognizition helix could potentially determine this phenotype by destabilizing the active conformation of DtxR relative to an inactive conformation in spite of binding to divalent cations, or by other mechanisms. Additional genetic and structural studies will be needed to define all of the ligands in DtxR that coordinate with the physiological activator Fe^{2+} or other bound divalent metal ions and

to explain fully the molecular basis for the allosteric changes in conformation of DtxR that occur as a consequence of its binding to metal ions..

III. Metal-Binding Domain of DtxR

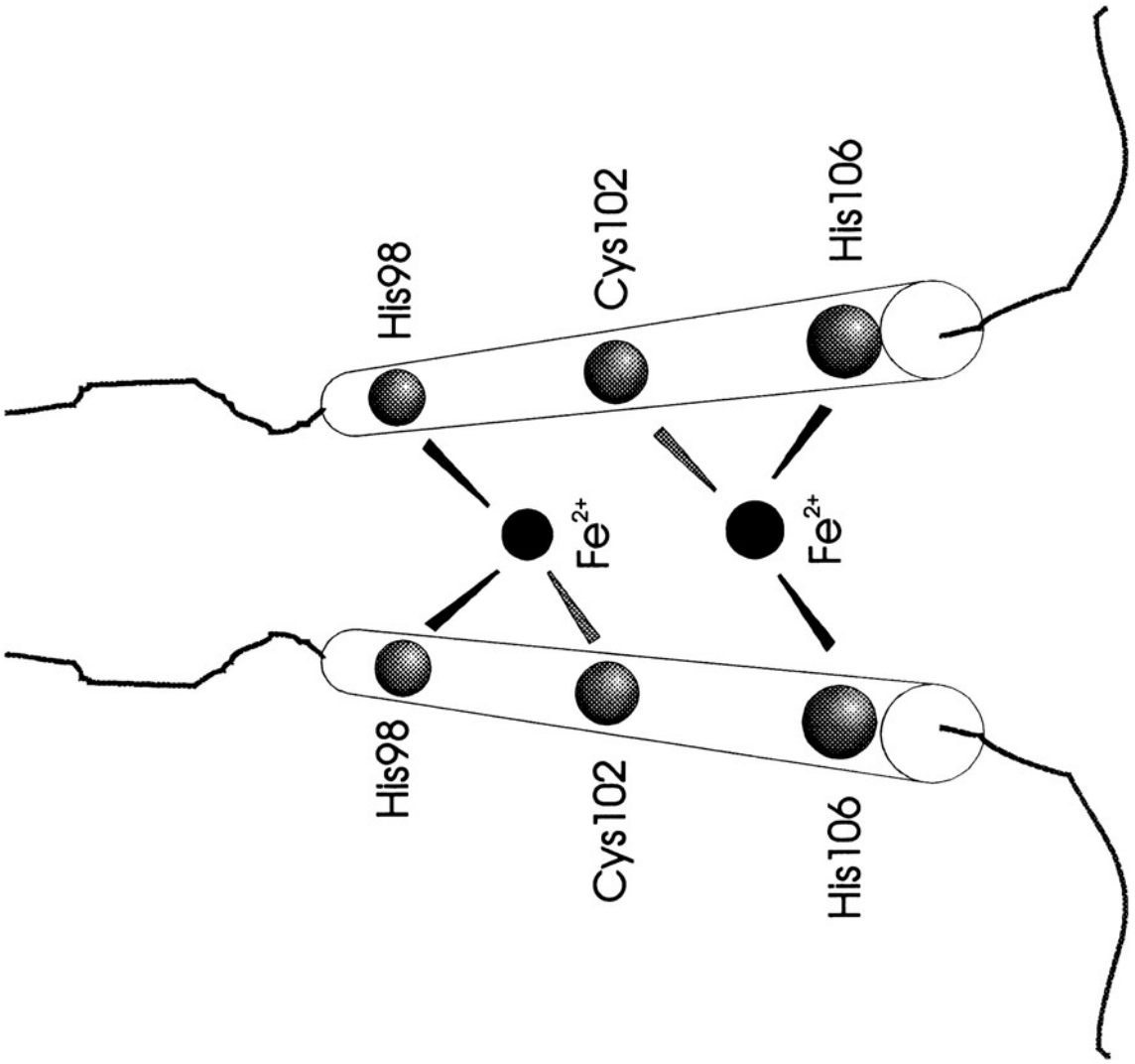
The metalloregulatory roles of histidine and cysteine residues in the conserved metal-binding sequence of DtxR (His98-X_3-Cys102-X_3-His106) were investigated in this report. Several amino acid substitutions for these residues were introduced into the conserved metal-binding sequence of DtxR. In vivo biological assays and subsequent in vitro DNA-binding and metal-binding assays revealed some interesting features of these conserved residues. Substitutions for the His-98 residue decreased the metal-binding activity of DtxR by approximately 50%, but did not substantively influence the normal metalloregulatory activity of DtxR. In contrast, substitutions for the His-106 residue also decreased the metal-binding activity of DtxR by approximately 50%, but resulted in the loss of repressor activity. Therefore, the ability of the residual divalent cation binding to activate repressor function was preserved in the His-98 variants but lost in the His-106 variants. These finding are consistent with the hypothesis that His-98 and His-106 are located in two independent metal-binding sites and that interaction of His-106 with a bound metal ion is important for activation of the DNA-binding activity of DtxR. Various amino acid substitutions for Cys-102 had different impacts on the DNA-binding and metal-binding activities of DtxR. Substituting histidine, another metal-coordinating ligand, for Cys-102 increased its activity in the Ni^{2+}-binding assay, and changed the DNA binding so that it was activated by Co^{2+}, Cd^{2+}, and Zn^{2+} but not by Fe^{2+}, Ni^{2+} or Mn^{2+}.

Although C102R and C102S exhibited only slight decreases in Ni^{2+}-binding activity, the DNA-binding activity of C102R was not activated by any of the metal ions tested, and that of C102S was fully activated only by Co^{2+} and Ni^{2+}. These findings suggested that the Cys-102 residue also has an important role in converting the transition metal signal into the regulatory function of DtxR. Amino acid substitutions within the putative DNA-binding domain of DtxR did not greatly affect the metal-binding activities of DtxR (Wang *et al.*, 1994). These findings support the concept that the transition metal signal is relayed from the divalent cation-binding site to the sequence specific DNA-binding site in the process of genetic switching controlled by DtxR.

IV. Model for the Metal-Binding Sites of DtxR

A preliminary model for the metal-binding sites of DtxR is presented in Figure 28. Residues His-98, His-106, and Cys-102 are principal ligands in forming two similar high-affinity, metal-binding sites in a functional DtxR dimer. The prominent features of this model are that: (i) the metal-binding sites are located at the interface between the two polypeptides of the DtxR dimer; (ii) the two metal-binding sites are situated symmetrically to the two Cys residues; (iii) the two His-106 residues together with one or both of the Cys-102 residues constitute one metal-binding site that is essential for the metalloregulatory function of DtxR; and (iv) the two His-98 residues together with one or both of the Cys-102 residues form a second metal-binding site which has comparable affinity for divalent metal ions but is not essential for activating the repressor activity of DtxR.

Figure 28. A schematic model of the metal-binding sites of the dimeric DtxR protein. The segments of each polypeptide chain including the His-98, Cys-102, and His-106 residues are shown. See text for more detailed description of the two putative inter-molecular metal-binding sites.


His98
Cys102
His106
Fe^{2+}
Fe^{2+}
His98
Cys102
His106

Results from several other experiments also favor this model for the structure-function relationships of DtxR. Evidence that DtxR is a dimer includes the direct demonstration of the nondisulfide-linked dimers of wild-type DtxR by in vitro crosslinking experiments and by HPLC gel filtration chromatography (S. Zhang and R. K. Holmes, personal communication), the dyad symmetry of the contacts of DtxR with its operators revealed by hydroxyl radical footprinting assays (Schmitt and Holmes, 1993 & 1994), and the dominant-negative phenotypes of some of the DtxR variants (Wang *et al.*, 1994). The easy formation of nonfunctional, disulfide-linked DtxR dimers indicates that the Cys-102 residues are surface exposed, that they are probably close to each other in functional DtxR, and that the free thiol groups of the cysteine residues are important for repressor function (Schmitt *et al.*, 1992; Tao *et al.*, 1993). The formation of crosslinked dimers of purified DtxR by the chemical crosslinking agent *Bis*maleimidohexane (BMH), which reacts only with sulfhydryl groups, also supports the surface exposure and neighboring location of the Cys-102 residues in a functional DtxR dimer (S. Zhang and R. K. Holmes, personal communication).

One prominent difference in metalloregulatory functions between DtxR and Fur protein, which is the iron-dependent regulatory protein in *E. coli*, is that Cu^{2+} can not activate DtxR to bind to DNA but can activate Fur protein in in vitro DNA-binding assays. Although the Fur protein has four cysteine residues and twelve histidine residues in its polypeptide sequence, only histidine residues close to the carboxyl-terminal end of Fur are considered to be involved in metal-binding activity, based on extensive proton nuclear magnetic resonance studies of Fur (Saito *et al.*, 1991a & 1991b). Because Cu^{2+}

can oxidize the free thiol group of cysteine, cysteine residues in metal-binding proteins are generally weaker chelators of Cu^{2+} than histidine residues are (Arnold and Haymore, 1991), and cysteines are only found in the metal-binding sites of a few copper-binding proteins (Ibers *et al.*, 1980; Ryden, 1989). The strong competition exhibited by Cu^{2+} in the competitive $^{63}Ni^{2+}$-binding assays with DtxR, which was very similar to the competition seen with other divalent cations, indicated that the failure of Cu^{2+} to activate DtxR was not due to its failure to bind to DtxR. Previous studies by Schmitt and Holmes (1993) also demonstrated that pre-incubation of DtxR with Cu^{2+} did not interfere with its subsequent metal dependent DNA-binding activity. Our studies also demonstrated that the Cys-102 residues of DtxR are not required for metal-binding activity, although the reduced cysteine residues are necessary for the metalloregulatory function of DtxR.

The variants containing the amino acid substitutions at position Cys-102 and His-106 showed dominant negative phenotypes in in vivo functional assays, which suggested that these variants could form nonfunctional heterodimers with the wild-type DtxR monomers. These results indicated that the metal binding activity which is essential for metalloregulatory function was not necessary for formation of DtxR dimers, even though the metal-binding sites of dimeric DtxR appear to be located at the interface of the two monomers. The location and structure of the dimer-forming domain of DtxR has not yet been determined and needs further study.

V. Subsequent Findings

After the experimental work and defense for this dissertation were completed, the

structure of DtxR in complex with several different divalent metal ions was determined (Qiu et al., 1995). These studies demonstrated that DtxR has two metal binding sites per monomer. In crystals of DtxR in complex with Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}, only binding site 1 was occupied. In crystals of DtxR in complex with Cd^{2+}, both sites were occupied. Site 2 was located at a potential hinge region between the interface domain and the DNA-binding domain and may be particularly important for the function of DtxR. The coordinationg ligands at binding site 1 are His79, Glu83, His98, and a water molecule that hydrogen bonds to Asn130. The coordinating ligands at binding site 2 are Cys102, Glu105, His 106 and a water molecule. The proposed location of the DNA-binding domain of DtxR was confirmed and was shown to be topographically related to the DNA-binding tomain of the catabolite gene activator protein CAP from *Escherichia coli.*

VI. Summary

1. The studies presented here identify several distinct regions in the amino-terminal half of DtxR that are required for repressor activity. Based on the phenotypes of our *dtxR* mutations, the properties of several purified DtxR variants, and similarities in amino acid sequence between DtxR and other repressors or divalent metal ion-binding proteins, motifs of DtxR that are presumed to be associated with operator-recognition and binding of divalent metal-ion activators have been identified.

2. I demonstrated the importance of residues His-98, Cys-102, and His-106 in metal-binding and metal-dependent DNA-binding of DtxR, and I constructed a model for

metal-binding by DtxR. Since none of the DtxR variants with substitutions for His-98 or His-106 had a total loss of high-affinity metal-binding, further studies of DtxR variants with substitutions for both of these metal-chelating histidine residues would be of interest. Direct evidence to rule out the possibility that other histidine residues or other amino acid residues are involved in metal-binding activity is also needed. Ultimately, determination of the geometry of the coordinated divalent cations in the metal-binding sites of DtxR by X-ray crystallography or other methods will be required to establish or disprove the model for metal-binding activity of DtxR presented here.

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